DISTAV
Dipartimento di Scienze della Terra, dell'Ambiente e della Vita
Università degli Studi di Genova

Ph.D. course of Science and technology for environment and territory
Curriculum: Marine Science
XXXI Cycle

Vibrios involved in *Crassostrea gigas* infections during mass mortality episodes

Coordinator: Prof. Luigi Vezzulli

Candidate: Dott. Giovanni Tassistro

INDEX

INTRODUCTION

Vibrio genus and aquatic ecology

The genus name *Vibrio* was coined by Pacini in 1854 during his study on cholera disease and it comes from these bacteria movement that appears like a vibration at microscope (Farmer III *et al.,* 2006). Vibrios are Gram-negative bacteria ubiquitary of the marine and estuarine waters, curved rod-shape 1.4-2.6 μm wide and motile by a single polar flagellum. They are facultative anaerobe capable of fermentative and respiratory metabolism, oxidase-positive, chiefly halophic and grow well at neutral and alkaline pH values up to 9 (Tantillo *et al.,* 2004; Igbinosa *et al.,* 2008). *Vibrio* species keep 2 chromosomes each of them with a distinct and independent origin of replication (Rasmussen et al., 2007). They are not able to form spore but they can enter the "Viable But Not Culturable" state (VBNC) as a survival strategy in the environment also for years (Colwell *et al.,* 1994; Lipp *et al.,* 2002). VBNC cells are in a dormant stage, are still alive but do not replicate (not culturable); however, they can re-growth if exposed to appropriate stimuli (Oliver *et al.,* 1991; Gauthier, 2000). An important aspect of this state is the possibility to maintain some virulence aspects: VBNC *Vibrio cholerae* O1 cells retain the ability to adhere to intestinal cells and, after reactivation, can cause disease (Colwell *et al.,* 1994).
Vibrio genus includes more than 100 species, some of them are pathogenic for humans (*V. cholerae*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*) others for marine invertebrates and vertebrates (*e.g.*, *Vibrio coralliilyticus* for corals, *Vibrio aestuarianus* for oysters) (Austin 2005; Paillard *et al.,* 2004; Thompson *et al.*, 2004a; Summer *et al.,* 2001; Chakraborty *et al.,* 1997).

Several *Vibrio* species have been isolated as natural component of some mollusc's microbiota, nevertheless they may also act as opportunistic pathogens *e.g.*, when environmental conditions are not optimal or when they infect an immune-compromised host (Saulnier *et al.,* 2010). *V. aestuarianus* and some species of *Splendidus* clade, ubiquitous in different geographic areas, such as USA, Europe, China and New Zealand (Tison and Seidler, 1983; Eiler *et al.,* 2006; Garnier *et al.,* 2008; Zhang *et al.,* 2011; Keeling *et al.,* 2014; Romero *et al.,* 2014; Scarano *et al.,* 2014), are associated with oyster mortality outbreaks; however, they have been also found in healthy and moribund *Crassostrea gigas* (EFSA 2015).
Vibrio spp concentration in the aquatic environment is related to different environmental variables, such as temperature, salinity, pH and presence of planktonic organisms. Most Vibrios prefer warm temperatures for growth as it is evident during Summer when their concentration drastically increases. Optimal salinity values are usually between 5 and 25/$_{00}$, but the tolerance can change between species (*V. cholerae* can survive from 0 and 45/$_{00}$ salt concentration) (Singleston *et al.,* 1982, Lipp *et al.,* 2002). Others environmental factors operate as limiting factors. For instance, iron is a fundamental nutrient for bacteria and algae in the water environment; *V. cholerae* can produce an iron-chelanting siderophores to take up insoluble iron from the environment (Payne and Finkelstein 1976).
A relevant feature that influences survival and concentration of Vibrios in the environment is the capability to interact with living and non-living substrates (Pruzzo *et al*., 2008). As usual for bacteria, Vibrios adhering to these substrates can survive longer in hostile environment (*e.g*., in

the presence of antibacterial factors or facing bivalve immunity system) than free-living form. Adhering bacteria can also participate to biofilm formation.

Biofilm

Most bacteria are capable of two general modes of growth, a planktonic or community-based lifestyle. In planktonic growth, organisms exist as independent, free-living cells, swimming or suspended in a liquid medium depending on their motility capacity (Boles and Horswill 2012). When environmental situations dictate, bacteria can grow together as a group and form biofilm structures, in which the cells live clustered in a matrix-encased community (Davey and O'toole 2000). Biofilm formation is commonly considered to occur in several stages:

1) Free-floating bacteria approach to a substrate (*e.g.* chitin for Vibrios) by random collisions or chemotaxis and adhere to the target.
2) Cells aggregate, form micro colonies and excrete extracellular polymeric substances (EPS), *i.e*. slime.
3) A biofilm is formed. It matures and cells form multi-layered clusters.
4) Three-dimensional growth and further maturation of the biofilm, providing protection against host defense mechanisms and antibiotics.
5) The biofilm reaches a critical mass and disperses planktonic bacteria, ready to colonize other surfaces.

The architecture of the biofilms include water-channels for the mobilization of the nutrients inside the structure and the toxic metabolites out of it. Commonly, biofilms are composed of mixed microbial species than a single species. In biofilms, bacteria display an exceptional resistance to environmental stresses, especially antibiotics. This makes biofilms a major public health problem as 60-80% of human microbial infections are caused by bacteria growing as a biofilm.

Interaction with aquatic substrates

Vibrio species can interact with various biotic and abiotic aquatic substrates to increase their survival in the water. Among the many substrates Vibrios can adhere to, the most common is chitin. Chitin, composed of β-1,4-linked *N*-acetylglucosamine (GlcNAc) residues, is one of the most abundant biopolymers in nature and, perhaps, the most abundant in the marine environment (Colwell, 2002). It is distributed throughout all kingdoms, as it is a crucial component of the cell walls of moulds, yeasts, fungi and certain green algae, and is a major component of the cuticles and exoskeletons of worms, molluscs and arthropods.

Binding to chitin is a complex process involving hydrophobic and ionic bonds, forces responsible for the primary reversible phase of attachment, and specific cell ligands that are responsible for subsequent firm anchoring to the substrate.

The presence of pili is associated with the ability of bacterial cells to colonize surfaces. Mannose-sensitive haemagglutinin (MSHA) is a type 4 pilus produced by *V. cholerae* and other vibrios, including bacteria pathogenic for bivalves (*e.g*., *V. aestuarianus*). In *V. cholerae* El Tor, a type 4 PilA-containing pilus, the expression of which is induced by chitin (also designated chitin-regulated pilus, ChiRP), has been reported to contribute to colonization of chitin (Meibom *et al.,* 2004). The toxin-co-regulated pilus (TCP), that is required for intestinal colonization and cholera toxin gene

acquisition by phage infection has also been shown to have a role in biofilm formation on chitin surfaces by *V. cholerae* (Reguera and Kolter, 2005).
Chitin binding proteins (CBPs) were found in several *Vibrio* species, both cell wall associated and extracellular (Montgomery and Kirchman, 1993; Pruzzo *et al.,* 1996; Tarsi and Pruzzo, 1999; Kirn *et al.,* 2005; Vezzulli *et al.,* 2008). In *V. cholerae* and other vibrios (*e.g., Vibrio alginolyticus, V. aestuarianus*), a 53 kDa protein, termed GlcNAc-binding protein A (GbpA), was found to be involved in GlcNAc-sensitive adhesion to chitin beads, *Tigriopus fulvus* (Zampini *et al.,* 2005) and *Daphnia magna* (Kirn *et al.,* 2005) plankton crustaceans, and to promote attachment to epithelial cell surfaces. These findings suggested that GbpA is important not only in the environment by augmenting *Vibrio* colonization of chitinous structures but also in the gastrointestinal tract by favouring their interactions with epithelial cells.

Chitin-*Vibrio* interaction has provided the microorganism with a significant number of properties, each linked one to the other, including cellular physiological responses (chemotaxis, chitin utilization, cell multiplication, induction of competence), multicellular organization (biofilm), and participation in carbon (C) and nitrogen (N) cycling in the aquatic ecosystem.
It has been shown that chitinolytic bacteria that are ubiquitous in the marine environment play a major role in chitin recycling in the ocean. Without bacterial activity returning the insoluble polysaccharide to the ecosystem in a biologically useful form, the oceans would be depleted of bioavailable C and N in a relatively short time. Adhering bacteria are able to metabolize chitin more efficiently than free-living cells, thereby increasing the rate of chitin mineralization in the natural environment. For complete hydrolysis of chitin to GlcNAc, the concerted action of chitinase and β-Nacetylglucosaminidase is essential. As all *Vibrio* species produce an active chitinase, it is suggested that a primary role of vibrios is the colonization of chitin and initiation of its degradation in aquatic ecosystems.

Several authors have proposed that plankton organisms and especially zooplankton (*i.e*. copepods) serve as marine reservoir of vibrios (*e.g., V. cholerae, V. aestuarianus*). These bacteria, adhering to external surfaces and the gut of crustaceans, can survive in seawater longer than free living planktonic cells (Colwell, 1996). Huq and colleagues (1983) first proposed that, once cells of *V. cholerae* attach to zooplankton, they are protected from the external environment and begin to proliferate, taking advantage of the increased surface area and improved conditions of nutrition. They also showed that *V. cholerae* associated with living copepods remained culturable at least 10 days or longer than *V. cholerae* associated with dead copepods, indicating that living copepods confer on vibrios some form of protection. Attachment to chitinous organisms, such as copepods and their eggs, which are dispersed in the water, or other aquatic living organisms, *e.g*. flying chironomids, ultimately provides the main mechanisms for dissemination and extended geographic distribution of *Vibrio* spp. (including pathogens) in nature.
It is noteworthy that a colonized copepod may contain up to 10^4 cells of *V. cholerae*; therefore, plankton blooms can provide the requisite infectious dose for clinical cholera when untreated water is ingested (Colwell, 1996). Nalin and colleagues (1979) also suggested that binding to chitin may offer protection for the vibrios from gastric acid during stomach transit when infecting humans. Thus, *Vibrio*–chitin interactions have also important consequences for pathogenicity for humans.

Bivalves and *Crassostrea gigas*

Bivalvia is the second largest class of *Mollusca* and it counts 9200 species of filter feeders. This class includes clams, oysters, cockles, mussels, scallops and numerous other families that live in saltwater, as well as a number of families that live in freshwater. The shell is made of calcium carbonate and enclose the animal body. The name *Bivalvia* comes from the division of the shell in two valves joined together along one edge (the hinge line) by a flexible ligament that, usually in conjunction with interlocking "teeth" on each of the valves, forms the hinge.

Crassostrea gigas is an oyster native to the Pacific coast of Asia. It has become an introduced species in North America, Australia, Europe, and New Zealand; it has solid shell, inequivalve and extremely fluted. As showed in Figure 1, the left or lower valve is deeply cupped and this distinctive tract granted it the name of Pacific cupped oyster (FAO 2014).

Figure 1. Oyster Crassostrea gigas shell. The right valve is flat while the left one is concave (Wikipedia)

As reported in 2014 FAO document, this oyster usually lives attached to rocks, debris or other oyster shells in different type of bottoms: from lower intertidal zone 40m to mud or sand-muds bottoms. *C. gigas* is a resilient animal that can survive in different environmental conditions: it can survive in a wider salinity spectrum from 10 to 35‰ salinities. Likewise, the Pacific cupped oyster has a wide temperature range of survivability, from -1.8°C to 35°C.

Pacific oysters are protandrous hermaphrodites, most commonly maturing first as males. In areas with good food supply the sex ratio in older oysters shows a predominance of females, whereas the reverse is true in areas of low food supply; females can revert back to male when food supply is limiting. Gametogenesis begins at around 10°C at salinities of between 15 and 32‰ and is rarely completed at higher salinities. Spawning generally occurs at temperatures above 20 °C and rarely at 15–18 °C. The species is very fecund with 8–15 cm length females producing between 50–200 million eggs in a single spawning (FAO 2014). Males and females of the Pacific Oyster release sperm and eggs into the water column. When the fertilization occurs, the embryogenesis starts to

develop the larvae that live in the water column. They measure 70 µm shell length and settle out of the water column to crawl, using the larval foot, to seek a suitable settlement location for attachment when 300–340 µm. This may take two to three weeks, depending on water temperature, salinity and food supply, during which time they can be dispersed over a wide area by water currents. As in other oyster species, mature Pacific cupped oyster larvae attach permanently to the chosen substrate by a cement secretion from a gland in the foot. Once settled they metamorphose into the juvenile form. Growth rate is very rapid in good conditions; market size being attained in 18 to 30 months.

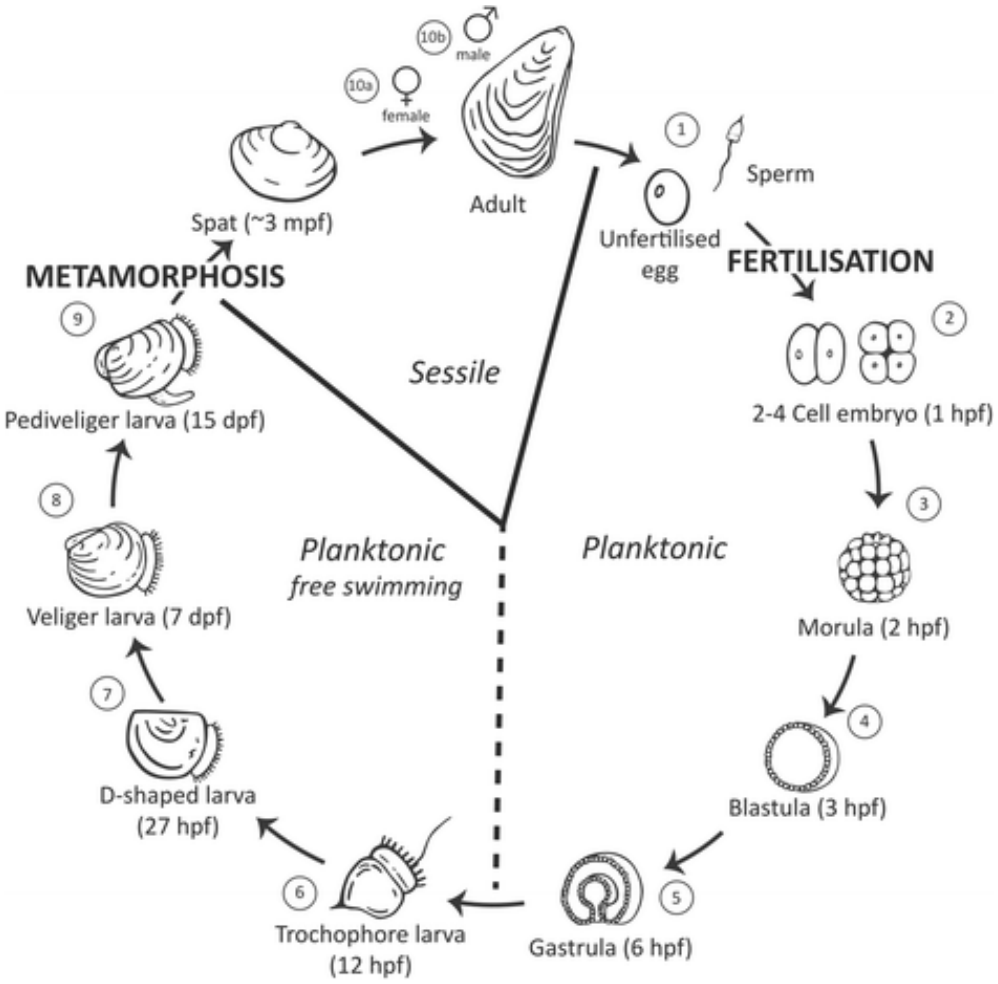

Figure 2. Crassostrea gigas life cycle. Numbers 1-10 represent sampling points for nuclear receptor expression analysis. hpf: hours post fertilisation; dpf: days post fertilisation; mpf: month post fertilisation (Vogeler et al., 2016).

The filtration mechanism of bivalves is an accurate system able to distinguish and filter the particles in the water column selectively, by dimension and shape. As shown in Figure 3, the gills act as a sieve of seawater, catching particles of optimum size and moving them toward the mouth, while particles that are too large are stopped and passed from the oyster as pseudofeces (Froelich *et al.,* 2013). Particles that are smaller than the optimum size pass through the gills without capture. These too are excreted from the oyster undigested. For *C. virginica*, the optimum particle size is 5 to 7 µm diameter, with particles of this size being retained with 90% efficiency. Particle retention rates drop to 50% when the diameter is only 1.8 µm, and when particles are the size of a

single *V. vulnificus* bacterium (ca. 1 µm) oysters retain only ca. 16% of what is passed through the gills (Ward and Shumway 2004; Froelich *et al.,* 2013). This size selection allows the filter-feeding to select the most attractive particles, like phytoplankton cells, and avoid or discard potential damaging elements, like planktonic bacteria cells, representing first system to avoid infection by avoiding free-living bacterial pathogen uptake.

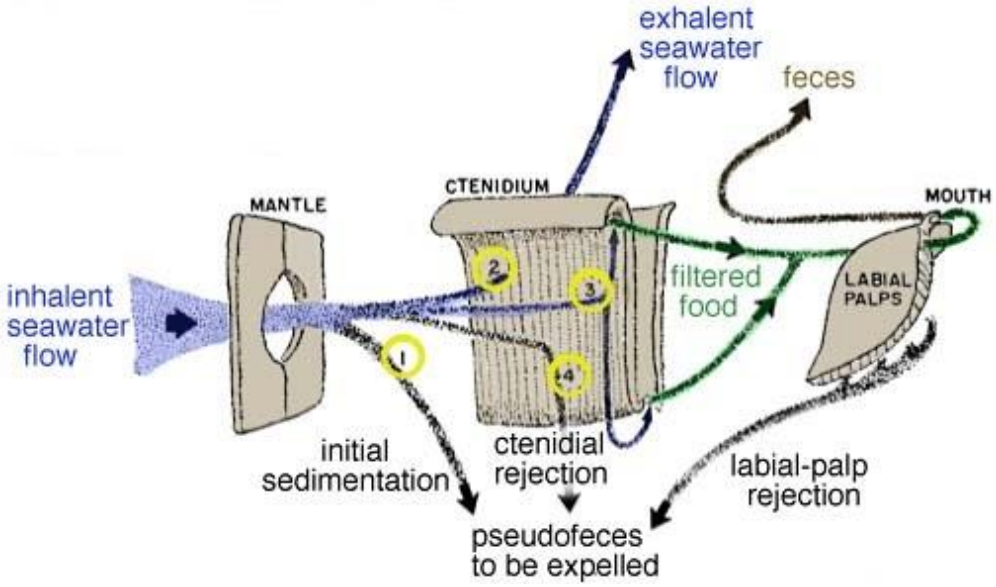

Figure 3. Diagram of water-flow paths and particle fates in oyster

The immune system of the *C. gigas* is able to protect the host from infections and its component can be found in hemolymph. There are two elements involved in this system: the hemocytes, cells responsible for cellular defense mechanism (*i.e.* phagocytosis, production of reactive oxygen intermediates and release of lysosomal enzymes) and humoral defense factors, such as opsonin and hydrolytic enzymes (Olafsen *et al.,* 1993). Some microorganisms are able to avoid the immune system mechanisms and infect the *C. gigas* leading to epidemics in some cases. Bacteria show different capacities to survive hemocyte phagocytosis as consequence of the different ability to attract phagocytes, interact with opsonizing molecules, bind hemocytes and survive intracellular killing (Canesi *et al.,* 2002).

C. gigas aquaculture

The molluscs cultivation is a worldwide economic activity, among them the main bivalve species cultured in the world are clam (36%), oyster (35%), scallop (14.6%) and mussel (14.4%). Global bivalves aquaculture production has increased over the last 20 years, recently slowed, reaching to 13 million tons per year that represents for a total value of US$13.8 billion. Along with fisheries, aquaculture ensures the income of 10–12% of the world's population (Rees J. *et al.,* 2010, FAOSTAT 2012).

Among oyster species, *C. gigas* is the most important cultured spp. that was introduced in west coast of United States from Japan in the 1920s and in France in 1966 (FAO 2014). The aquaculture

of this spp. represents 97% of the oysters worldwide production (mainly from China, Japan, Republic of Korea and France) followed by *Ostrea edulis*.

In the lasts 20 years, significant mortality outbreaks of farmed *C. gigas* have been reported in many parts of the world: France, Spain, Netherlands, United Kingdom, Ireland, United States, Australia and New Zealand (Samain and McCombie 2008; EFSA 2015; Go J. *et al.*, 2017).

These annuals events occurring during summer season, called "summer mortality", affect both spat and adults animals (Samain and McCombie 2008). These recurrent outbreaks have been linked with a combination of different factors (physiological condition, rearing techniques, environmental conditions, genetic predisposition and specific pathogens) that acted synergistically, as summarized in Figure 4 (Samain and McCombie 2008).

Since 2008, the pattern of these mortalities outbreaks has changed with higher mortality and more widespread geographic distribution than previously seen (FAO 2014).

Although specific microbial pathogens (*i.e*. OsHV-1 and *V. aestuarianus*) have been identified to play a role in oyster diseases (Travers *et al.,* 2015) there is an emerging view that microbial infections may derive from the contribution of different microbial species/strains that act as a "community of pathogens" rather than a single species/strain as the only etiological agent (Lemire et al., 2015). Under this perspective evidence has been provided supporting the view that oyster infections might be seen as infectious disorders caused by the contribution of a larger number of pathogens (*e.g*. populations or consortia) than previously thought (Lemire et al., 2015).

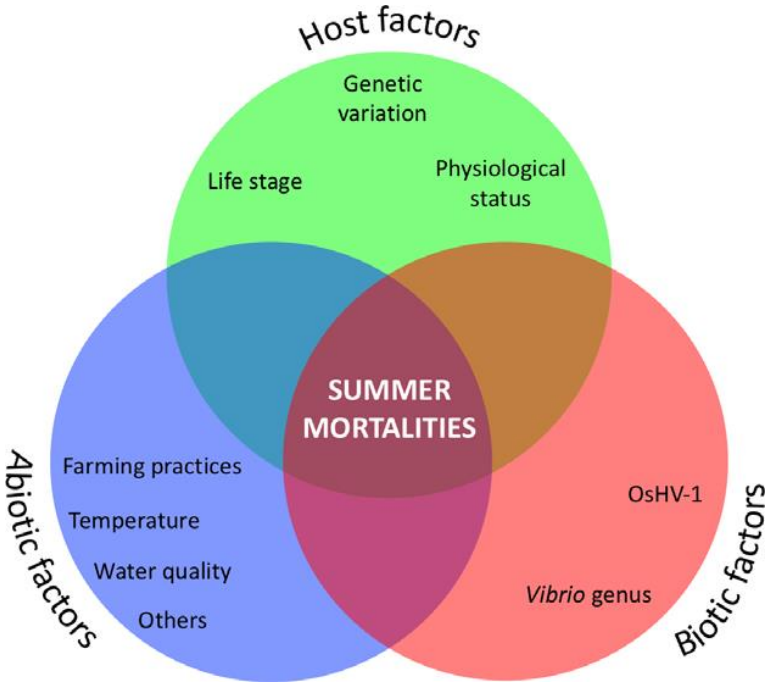

Figure 4. Venn diagram of main factors that contribute to mass mortality (Alfaro et al., 2018)

Bacterial factors

Among the different biotic factors with incidence on massive oyster mortalities, Vibrios are one of the main biotic factors of these outbreaks. Several species have been isolated during these events and associated to mortalities: *V. aestuarianus* and the different species of *V. splendidus* clade were the most common, although other *Vibrio* spp. were isolated such as *V. harveyi*, *V. tubiashii* and *V. alginolyticus* (summarized in Alfaro *et al.,* 2018).

Some *V. aestuarianus* strains were identified as the main bacteria pathogens causing *C. gigas* mortalities in different European aquaculture settings. Although it could affects oysters at all development stages, adults are more susceptible to be infected by this bacteria spp. rather than spat (Garnier *et al.,* 2007; Saulnier *et al.,* 2010; Azema *et al.,* 2016). Within this pathogen two different subspecies have been identified: *V. aestuarianus* subsp. aestuarianus and *V. aestuarianus* subsp. francensis, however only the latter has been associated to outbreak events (Garnier *et al.,* 2008). *In vitro* infection tests of *C. gigas* with different strains of *V. aestuarianus* subsp. francensis exhibit variable virulence among them. The experimental challenge was carried out *in vitro* infection experiments by intramuscular injection of bacterial suspensions, oyster immersion in contaminated water with pathogen or cohabitating injected oysters with healthy oysters (summarized in Alfaro *et al.,* 2018).
Among the 12 virulent strains found, one of the highly virulent strains resulted *V. aestuarianus* subsp. francensis 02/041 with 80% of mortalities by injection of 100 bacteria/animal (Goudenège *et al.,* 2015). Goudenège *et al.,* (2015) suggests that the strains can be classified by their potential virulence: from highly virulent (>50% mortality of oysters at 10^2 CFU/animal) to intermediate (strain pathogenic only at 10^7 CFU/animal) and non-virulent (<50% mortality at 10^7 CFU/animal). These virulence variations could be explained by the acquisition of virulence genes by horizontal gene transfer inducing a different virulent lineage (Le Roux *et al.,* 2011).
It has been suggested that infected animals can release up to 10^5 bacteria in 24h and promote disease-spread in a susceptible population (Travers *et al.,* 2017).

Several virulence factors were identified in Vibrios associated to pathogenicity for human or animals. The main one of *V. aestuarianus* linked with his pathogenicity against *C. gigas* is a zinc-metalloprotease "*vam*". This enzyme affects protein structures required for oyster hemocytes to mantein the morphology and develop a phagocytic activity, allowing *V. aestuarianus* to survive and replicate within the host (LaBreuche *et al.,* 2010).
Besides, some spp. belonging *Splendidus* clade have been found associated to oyster mortalities. It has been demonstrated that *V. tasmaniensis* LGP32 strain can invade oyster hemocytes by altering the cytoskeleton and surve within the host intracellular (Duperthuy *et al.,* 2011). Moreover this strain can secrete a high toxic metalloprotease (*vsm*) associated with pathogenicity on Pacific Oyster when injected in the animals (Le Roux *et al*., 2007).
Also the co-presence of both bacteria could increase their virulence by quorum sensing gene expression induction: *i.e. in vitro* test showed that the production of autoinducer-like substance by *V. tasmaniensis* LGP32 has intra- and interspecific effects on the expression of two different metalloprotease genes: *vsm* and *vam* (De Decker *et al.,* 2013).

It is demonstrated the key role of *V. aestuarianus* in *C. gigas* mortalities events in farming areas but also other Vibrios were associated to these events although their role in these events are not clear yet. It has been suggested that they can act as opportunistic pathogens under adverse conditions (Azandegbe *et al.,* 2010; Domeneghetti *et al.,* 2014; Vezzulli *et al.,* 2015), they have a primary role as a pathogen (Petton *et al.,* 2015b), or they actins together with other spp. as "community of pathogens" (Lemire *et al.,* 2015).
As reported above and shown in Figure 5, *Vibrio* spp., including species associated to *C. gigas* outbreaks, can adhere and accumulate on both chitin surfaces and marine organisms (Vezzulli *et*

al., 2014). These aquatic substrates, like marine snow (a shower of organic material falling from upper waters to the deep ocean), chitin particles and zoo/phytoplankton, may accumulate large communities of bacteria including oyster pathogens (*e.g.*, *V. aestuarianus* or *Splendidus* clade spp.). Inside the community, pathogens can increase their virulence potential by quorum sensing, enhancing gene expression of virulence factors. In addition, colonized substrates when filtered by the bivalve may introduce inside the animal high amount of pathogens favouring the infection.
It has been demonstrated that the human pathogen *V. cholerae* can reach concentration of 10^3 – 10^5 on a single copepod; since the requisite infectious dose for clinical cholera is 10^6 bacteria/ml, ingestion of untreated water containing colonized copepods could initiate the disease (Cash *et al.,* 1974; Colwell *et al.,* 1992).

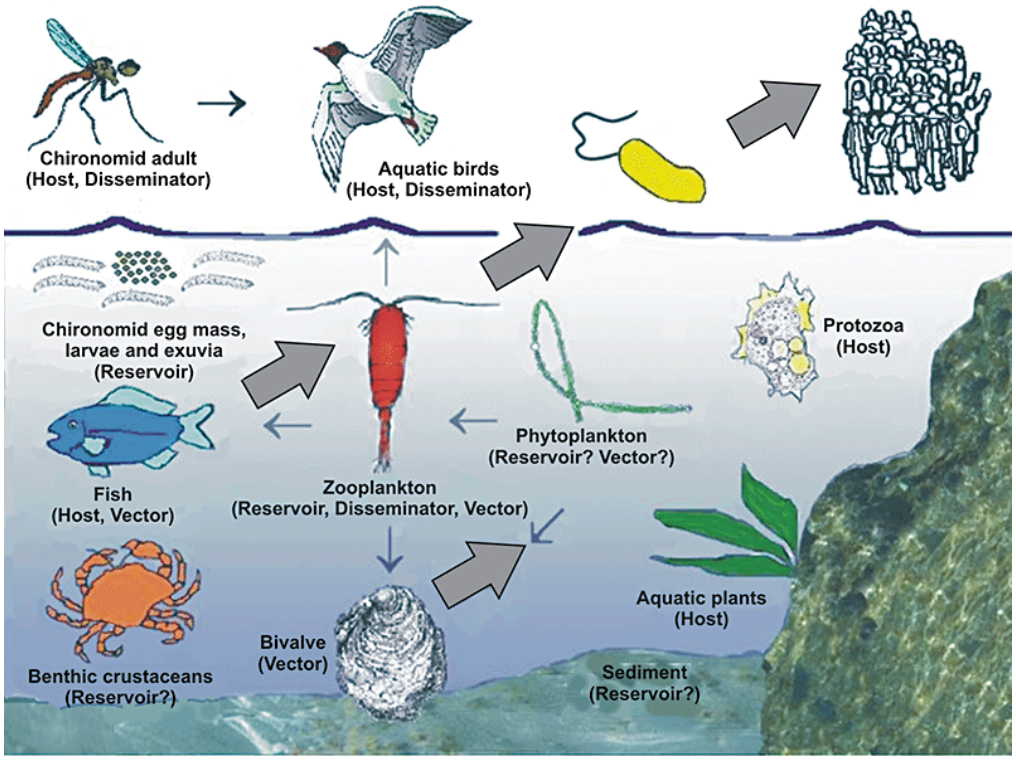

Figure 5. Variety of reservoir and vector of V. cholerae in the marine environment (Vezzulli et al., 2010)

Ostreid herpesvirus 1 (OsHV-1)

Herpesvirus and herpes-like virus infections have been related with mortalities of several bivalve mollusc species, from larvae stage to adult. Among them, some species have been reported to experience high mortalities, including Pacific oyster (*C. gigas*), European flat oyster (*O. edulis*), the Manila clam (*Ruditapes philippinarum*), the great scallop (*Pecten maximus*), the Portuguese oyster (*Crassostrea angulata*), the blood clam (*Scapharca broughtonii*) and the Chinese scallop (*Chlamys farrieri*) (Le Deuff and Renault 1999; Friedman *et al.,* 2005; EFSA 2015; Alfaro *et al.,* 2018).
OsHV-1 has high genetic diversity indeed several variants were isolated and linked during Pacific oyster outbreaks. Since 2008, in Europe only the OsHV-1 µVar has been isolated from mortalities in *C. gigas* aquaculture, usually found associated to juvenile mortalities (EFSA 2015; Degremont *et al.,* 2015).
Similarly for the *V. aestuarianus* infection, it is demonstrated that OsHV-1 infected oysters release the pathogen in the environment allowing the transmission into other organisms. The proximity of

hosts has a key role in the mortality rate, due to low survival time of free-living virus in the aquatic environment (Pernet *et al.,* 2012; Alfaro *et al.,* 2018).

Abiotic and biotic factors interaction

The mortalities are complex events where several factors act in concert to impair oyster's health conditions, as showed in Figure 4.

The host physiological status or the stressful environmental factors can affect the *C. gigas* health and make the bivalves more sensitive to pathogen infections. Salinity have positive relationship with *Vibrios* abundance in the marine environment (Hsieh *et al.,* 2008).

Despite no studies have not found a link between the seawater temperature and the ability of *V. aestuarianus* to infect oysters, the mortality outbreaks associated with this bacteria occur mainly in the summer, when the temperature is 19-20°C, near to the optimal growth temperature of *V. aestuarianus* (Paul-Pont *et al.,* 2014; Vezzulli *et al.,* 2014). Moreover, the increase of the water temperature influence also the OsHV-1 virulence increasing the mortality rate of the Pacific oyster (De Kantzow *et al.,* 2016). When the temperature decrease under 13°C also the mortality rate decrease (Garnier *et al.,* 2007; Azandegbe *et al.,* 2010; Dégremont *et al.,* 2014a).

Not only the environmental conditions can drive these mortalities but also the host physiological condition: like the sexual maturity is connected with higher susceptibility to *Vibrio* infections (De Decker *et al.,* 2011) or the ploidy, triploid juveniles are more susceptible to *V. aestuarianus* infection (Azemà *et al.,* 2016).

Considering the interaction between the *C. gigas* pathogens with planktonic elements of the sea, the environmental conditions can influence the outbreaks also in a secondary way. Temperature and salinity control the growth of phytoplankton and aquatic plants, diet of zooplankton and organisms with chitin exoskeletons, potential reservoir or/and vehicle of *V. aestuarianus* and OsHV-1 (Cockburn and Cassanos, 1960).

C. gigas microbiome

Mass mortality episodes of the Pacific Oyster in European farming areas are attributed to complex interactions among host physiological conditions, microbial pathogens and environmental variables (Lemire *et al.,* 2015; Alfaro *et al.,* 2018). Usually high microbial diversity is index of a healthy oyster while diseased organisms present a lower microbiome diversity (Lokmer & Wegner 2015; Clerissi *et al.,* 2018). In fact, stressful environmental conditions such as warm seawater temperatures was observed to favour shift of *C. gigas* bacterial communities toward pathogen-dominated communities, also promoting colonization by secondary opportunistic pathogens (Lokmer & Wegner 2015).

A recently study evaluate the microbiome of *C. gigas* and *Mytilus galloprovincialis* from the Italian aquaculture in the Gulf of La Spezia finding high relative abundance of Vibrios in hemolymph and digestive gland. Moreover, the different elements of the bivalves showed different microbiomes: in hemolymph it is more variable, influenced from external environment, while in the digestive gland is more stable (Vezzulli *et al.,* 2018). It is already confirmed that increasing seawater

temperature effect *C. gigas* by involving microbiome in pathogen-shift or promoting secondary opportunistic pathogens (Lokmer & Wegner 2015).
Filter-feeding organisms like bivalves can therefore accumulate a diverse microbiome including potential opportunistic pathogens that can persist and growth within the host, until production of disease and resulting outbreaks (Paillard *et al*., 2004; Garnier *et al*., 2007; Vezzulli *et al.,* 2018). In this light the knowledge of microbiome is an important step for understand the infection vulnerability and the disease progression.

OBJECTIVES

My PhD project was focused on the study of *C. gigas* outbreak events facing two different issues.
First, based on previous studies suggesting that certain plankton species are associated to the transmission of pathogenic vibrios to humans (Colwell *et al.,* 2003, Rawlings *et al.,* 2007), I studied if plankton might constitute an important driver of pathogen transmission to oysters too. To this end, I carried out microcosm experiments using *V. aestuarianus* 02/041, the main bacterial pathogen linked to oyster mortalities. Oysters were exposed to bacteria in the presence or in the absence of marine plankton elements, and their response to the different infection conditions was evaluated by hemocyte lysosomal stability test that represents the most sensitive biomarker of oyster health status (OSPAR, 2013).
In the second part of my thesis work, I studied the microbial communities associated to contrasting *C. gigas* samples collected during mortality episodes in different European sites with the aim to shed light on the structure and presence of recurrent patterns in bivalve microbiota during abnormal mortality episodes. A new target enrichment next generation sequencing protocol was developed and applied for the first time which allowed high taxonomic resolution analysis of the *Vibrio* and pathogen microbial community associated to the bivalve tissues.

Evaluation of planktonic substrates as potential infection vectors of *V. aestuarianus* 02/041 in pacific oyster *C. gigas*

Objectives

Several studies have shown that *V. aestuarianus* and bacteria belonging to *Splendidus* clade (*e.g*. *V. tasmaniensis* LGP32) are associated with mortalities affecting the production of *C. gigas* worldwide. However, knowledge is still missing on the environmental factors driving these pathogen dynamics and potentially favoring their transmission to oysters.

In the frame of the BIVALIFE FP7 EU project (2011-2014) it was shown that both *V. tasmaniensis* LGP32 and *V. aestuarianus* 01/32 are able to interact with chitin particles and plankton crustaceans (e.g., *Tigriopus fulvus* copepods) present in the aquatic environment via specific ligands such as the mannose-sensitive haemagglutinin (MSHA) pilus and the N-acetylglucosamine binding protein A (GbpA). Based on these results it was suggested that the above substrates might represent important reservoirs for *Vibrio* bacteria in the marine environment (Vezzulli et al., 2015). Interestingly, plankton species have previously been associated to the transmission of pathogenic vibrios to humans (Colwell *et al.,* 2003, Rawlings *et al.,* 2007). Therefore, in this work, we studied if plankton substrates might constitute an important driver of pathogen transmission to oysters too.
To investigate this hypothesis, laboratory experiments were conducted to study whether different representative marine substrates *i.e.* phytoplankton cells (*Nannochloropsis gaditana*), marine snow particles and chitin fragments might represent suitable infection vehicle for strains pathogenic for the Pacific oyster *C. gigas.*
To mimic bivalve exposure to microbial pathogens under natural environmental conditions, oysters were exposed to pathogenic bacteria by immersion, both in the presence or in the absence of plankton* substrates. The presence and concentration of bacterial pathogens in the bivalve hemolymph was evaluated two days post infection; hemocyte lysosomal stability tests were performed to evaluate oyster's health status. *V. aestuarianus* 02/041, a virulent strain for the Pacific oyster *C. gigas,* was used as model in this study.

Another objective of my work was to study whether *Vibrio* interactions with specific plankton substrates may also affect their virulence and expression of pathogenicity related properties, thus playing a role in the outcome of diseases in the Pacific oyster *C. gigas*.
Vibrio pathogenicity and virulence towards bivalves have been partially linked to the production of extracellular products (ECPs) and, in particular, to extracellular proteases. For example, in *V. aestuarianus*, a zinc metalloprotease (designated as Vam) was identified and was shown to induce immunosuppressant activities on *C. gigas* hemocyte functions *in vitro* most probably through the modulation of hemocyte cell physiology (Labreuche *et al* 2010).
In this work, the effect of interaction of different *Vibrio* strains involved in bivalve diseases (*V. aestuarianus* 02/041, *V. tasmaniensis* LGP32, *V. coralliilyticus* ATCC BAA450, *V. harveyi* VH2, *V. tapetis* CECT 4600) with representative plankton substrates (*i.e*. marine snow particles and chitin fragments) on protease activity was evaluated *in vitro*. To this end, extracellular aminopeptidase

activity of the strains was measured through fluorogenic assays in the presence and in the absence of the different substrates.

*From here on in the text, the term “plankton” was considered in more broader term *i.e*. as living and non living substrates floating in the water column which might potentially be filtered by *C. gigas* (avergage size <100um) in its natural farming environment.

Bacteria and culture conditions

The virulent *V. aestuarianus* 02/041 strain isolated during an oysters mortality outbreak (Goudenège *et al.*, 2015; De Decker and Saulnier 2011) was used in the infection experiments. *V. aestuarianus* 02/041, *V. tasmaniensis* LGP32, *V. coralliilyticus* ATCC BAA450, *V. harveyi* VH2 and *V. tapetis* CECT4600 strains were used to study the production of protease in the presence and in the absence of plankton substrates.

Bacteria were cultured overnight under constant shaking at 24°C in Luria-Bertani (LB) broth (Scharlau, Italy) containing 3% NaCl. Cells were centrifuged at 4500 g for 10 min and the resulting pellet was washed twice in PBS (0.1 M KH2PO4, 0.1 M Na2HPO4, 0.15 M NaCl, pH 7.2 to 7.4), and resuspended in Artificial Sea Water (ASW) (31ppt or 15ppt salinity) to the concentration of about $1x10^{10}$ CFU/ml. Thiosulfate Citrate Bile salts Sucrose (TCBS) agar (Scharlau, Italy) was also used for isolation of *Vibrio* colonies.

Oysters stabulation

Adult *C. gigas* oysters originating from Bay of Biscay (La Rochelle, France) length 8-10 cm and weight 71.7-116.4 gr were taken from local market. The bivalves were well cleaned from any epibionts and washed with fresh ASW (salinity 31ppt) at 18°C. After the cleaning, the oysters were stabulated for 24 hours in aerated 2 L jars without flow-through containing ASW 31ppt at 18°C. Each *C. gigas* were kept individually to avoid bacterial transfer among the oysters (Azema *et al.,* 2016).

Vectors for oyster infection

Substrates tested as potential vectors of *V. aestuarianus* were: phytoplankton cells (*Nannochloropsis gaditana*), marine snow particles and chitin fragments.

Phytoplankton cells

Microalgal culture of *N. gaditana* has been prepared in Walne medium (Walne, 1966) and kept in aerobically conditions at 25°C in the presence of the light. After 7 day incubation the exponentially growing cells were washed by centrifugation at 4500g for 10 minutes and the resulting pellet was resuspended in ASW (salinity 15ppt or 31ppt) to a final concentration of about 4-6 x 10^7 cells/ml, as evaluated by microscopy examination.

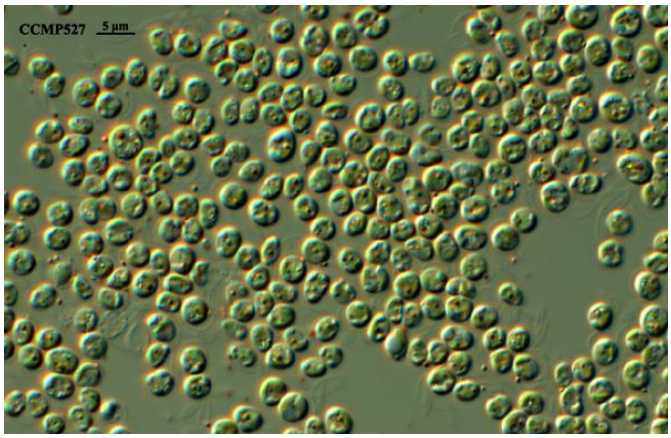

Figure 6. N. gaditana cells (photo from Provasoli-Guillard National Center for Marine Algae and Microbiota)

Marine Snow

Commercial Marine Snow (Two Little Fishies, Miami USA), consisting of fragments (0.2 to 150 µm sized) of phytoplankton (*Nannochloropsis, Tetraselmis, Isochrysis, Spirulina, Schyzochitrium*) and zooplankton organisms (as specified by manufacturer instructions), was used (approximately 4-6 x 10^7 particles/ml, as evaluated by microscopy examination).

Chitin fragments

Shrimp shell chitin (Sigma-Aldrich) was resuspended in ASW (salinity 15 ppt) and treated with a potter homogenizer. The resulting suspension was filtered on a 100 µm net in order to select beads suitable for oyster filtration (Froelich *et al.,* 2013; Kamiyama 2011), and sterilized by autoclave. After centrifugation at 4500 g for 10 min, the resulting pellet was washed twice in ASW (salinity 15ppt) and resuspended in ASW (salinity 15 ppt or 31 ppt) to a final concentration of about 4-6 x 10^7 chitin fragments /ml (as evaluated by microscopy examination).

Preparation of suspensions of *V. aestuarianus* 02/041 bacteria associated with plankton vectors

Suspensions of bacteria associated with different potential plankton vectors (*N. gaditana* cells or marine snow particles or chitin fragments) were prepared by inoculating 0.5 L jars containing 350 ml ASW (15ppt or 31ppt salinity) with *V. aestuarianus* 02/041 bacteria (final concentration about $1x10^8$ bacteria/ml) plus potential vectors (final concentration about $1x10^5$/ml *N. gaditana* cells, or marine snow particles or chitin fragments). After 2 hour incubation at 18°C, to allow interactions of bacteria with potential vectors, the suspensions were used to infect oysters or to evaluate endopeptidase activity (see below).

Oyster infection by immersion and hemolymph extraction

Oysters were challenged by immersion with *V. aestuarianus* 02/041 associated/non-associated with the different potential plankton vectors (*N. gaditana* cells or marine snow particles or chitin fragments); controls with the potential vectors alone were always included. Briefly, 0.5 L jars containing 350 ml ASW (salinity 15 ppt or 31 ppt) were inoculated with either bacteria alone (final concentration about $1x10^8$ bacteria/ml), or potential vectors alone (final concentration about $1x10^5$ *N. gaditana* cells (or marine snow particles or chitin fragments)/ml, or bacteria plus potential vectors as described above (paragraph 4); for each condition at least triplicate jars were used. Single oyster was then added to each jar and exposed to infection for 4 h at 18°C. Oysters were then removed from the jars, placed into clean 2 L jars containing fresh ASW (salinity 15 ppt or 31 ppt) and incubated at 18°C for further 2 days. Each oyster was then removed from experimental jars, rinsed with ASW and patted dry with paper towels. Hemolymph was extracted from the posterior adductor muscle using a sterile 1 ml of syringe with an 18 G1/200 needle. Controls with uninfected oysters were always included to evaluate background population count of *V. aestuarianus* experiments were performed in triplicate (at least 9 oysters tested per condition).

Enumeration of culturable vibrios in hemolymph

Hemolymph extracted from each single oyster (see above) was serially diluted in sterile PBS and spread onto TCBS agar plates. After 24 h incubation at 24°C, colonies of *Vibrio*-like bacteria were

counted; results were expressed as CFU per ml and represent an average of triplicate or quintuple samples. For the identification of *V. aestuarianus*, DNA was extracted from selected colony morphotypes by boiling (10 min, 99°C) and analyzed by species specific real-time PCR assay.

Enumeration of total *V. aestuarianus* and *Vibrio* spp. bacteria by real-time PCR

DNA was extracted from hemolymph of each single oyster using the High Pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Real-time PCR for total *V. aestuarianus* and *Vibrio* spp. cell counting was performed using LightCyler (Roche Diagnostics) with a Taqman or a BYRT Green real-time PCR protocol. For *Vibrio* spp. enumeration, genus-specific primers (F-GGCGTAAAGCGCATGCAGGT; R-GAAATTCTACCCCCCTCTACAG) (Thompson *et al*., 2004) were used following conditions described in Vezzulli *et al.* (2015). Real-time PCR for the enumeration of *V. aestuarianus* was performed using specific primers and probe (DNAj F GTATGAAATTTTAACTGACCCACAA; DNAjR CAATTTCTTTCGAACAACCAC; DNAj probe FAM–TGGTAGCGCAGACTTCGGCGAC – BHQ2) (Saulnier *et al*., 2009) were used following conditions described in Vezzulli *et al.* (2015). Accurately quantified copy number of genomic DNA of *V. aestuarianus* 02/041 was used as a standard. Results are expressed as an average of triplicate or quintuple samples.

Evaluation of hemocyte lysosomal membrane stability

Lysosomal membrane stability (LMS) was evaluated by the neutral red retention time (NRRT) assay as previously described (Balbi *et al.*, 2017). Briefly, hemolymph from 3-5 oysters was pooled, hemocyte monolayers were prepared on glass slides, washed out and incubated with 30 ml of a neutral red (NR) solution (final concentration 40 mg/ml from a stock solution of NR 20 mg/ml dimethylsulfoxide). After 15 min, excess dye was washed out, 30 ml of ASW was added, and slides were sealed with a coverslip. Every 15 min, slides were examined under optical microscope and the percentage of cells showing loss of dye from lysosomes in each field was evaluated (an example is shown in Figure 7. For each time point, 10 fields were randomly observed, each containing 8 -10 cells. End point of the assay was defined as the time at which 50% of the cells showed sign of lysosomal leaking, i.e. the cytosol becoming red and the cells rounded. Triplicate preparations were performed for each sample. All incubations were carried out at 18°C.

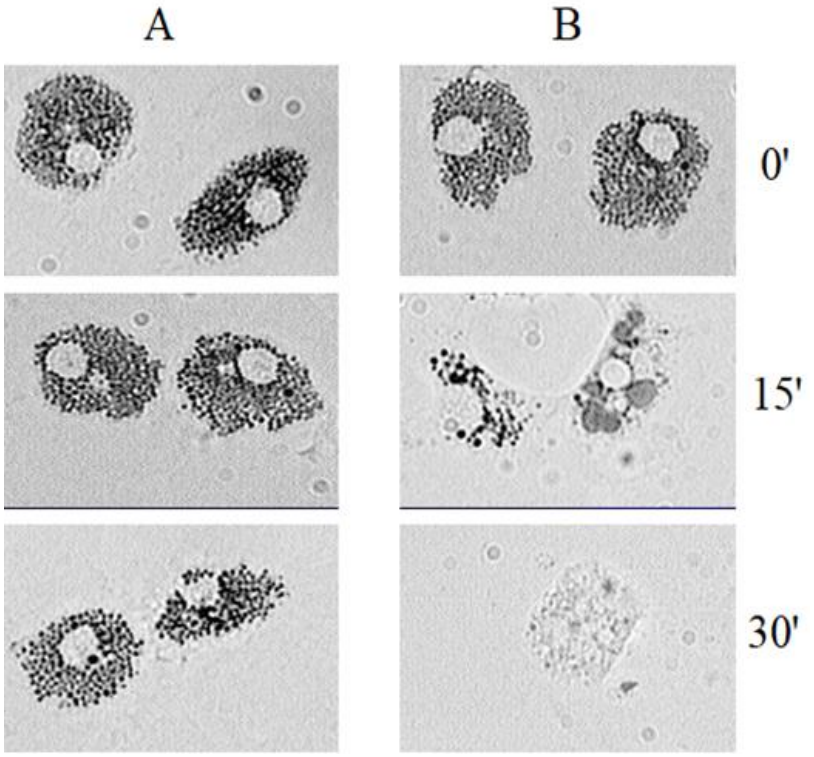

Figure 7. M. galloprovincialis hemocyte health status as shown by LMS assay. A: control hemocytes; B: hemocytes treated with a stressful agent. After 15 min incubation, neutral red (black dots) is released by stressed hemocytes while control cells keep the dye.

Evaluation of aminopeptidase production by associated and non-associated bacteria

Suspensions of bacteria associated and non-associated with *N. gaditana* cells or marine snow particles or chitin fragments were prepared as described above (par. 4). Controls consisting of plankton substrates alone were also included (Figure 8). After 2 hours incubation at 18°C, 4.5ml of the suspensions containing plankton vectors and associated bacteria, were inoculated with 0.5 ml of L-leucine-4-methylcoumarinyl-7-amide (Leu-MCA, final concentration 500uM). Samples were then incubated for 1 h (enzymatic activity increased linearly with time up to 3 h) in the dark at 18°C and enzymatic rates were assessed by fluorometric analysis (at 380 nm excitation, 440nm emission for Leu-MCA (Meyer- Reil, 1986) and corrected by subtracting the corresponding control values.

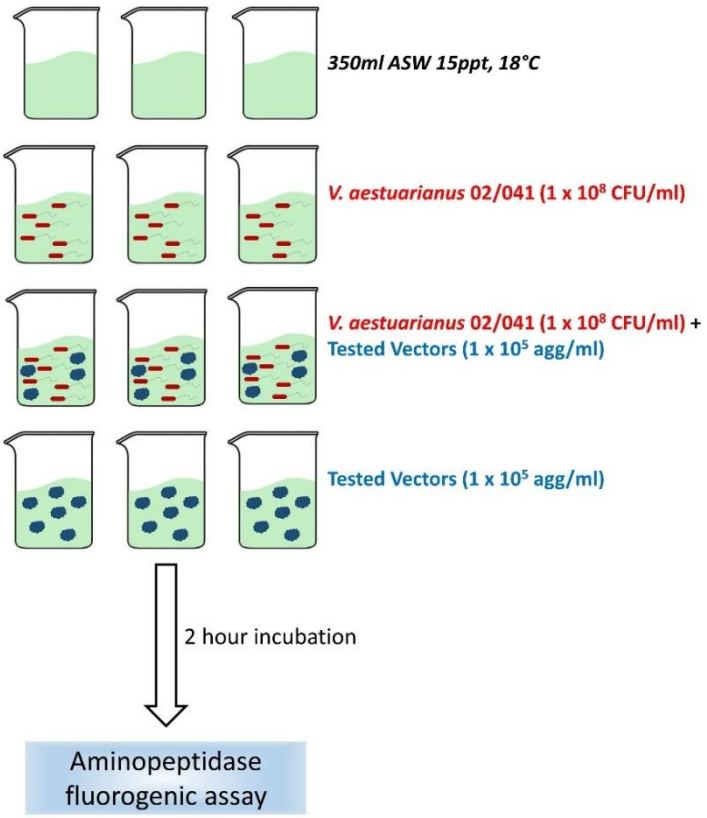

Figure 8. Experimental set-up for aminopeptidase activity assays

Evaluation of V. aestuarianus interactions with marine snow and chitin by Scanning Electron Microscope (SEM)

Suspensions of bacteria associated with plankton were prepared as described above (par. 4) with the difference that *V. aestuarianus* and plankton final concentrations were 4 x10^7 CFU/ml and 4x10^4 marine snow particles (or chitin fragments)/ml, respectively. Controls with bacteria or plankton alone were included. After 2 hours of incubation at 18°C the samples were filtered by vacuum pump on 0.2 polycarbonate filter (GTBP02500, Millipore). Specimens retained on the filter were fixed with 2.5% glutaraldehyde solution for 20 minutes and subjected to dehydration by ethanol series (25%, 40%, 60%, 80%, 90% and 100%, each step 10 min). Samples were dried and mounted onto the microscope stage, coated with gold and finally viewed using a Vega3 (Tescan) SEM.

Results

Preliminary tests

Evaluation of *V. aestuarianus* association to potential vectors

Before performing infection experiments with *V. aestuarianus* 02/041 associated to potential plankton vectors, we measured the efficiency of bacterial interaction with the different substrates. Two types of experiments were set up.

First, we evaluated the number of bacteria associated to plankton. To this end, suspensions consisting of bacteria plus plankton (*N. gaditana* cells or marine snow particles or chitin

fragments) (see Materials and Methods, paragraph 4) were prepared and, after 2 h incubation, were centrifuged (1000 g, 5 min). Supernatants were kindly removed and the resulting pellets, including plankton vectors and associated bacteria, were used for DNA extraction and enumeration of *V. aestuarianus* bacteria by PCR. On average, about 0.1% of bacteria inoculated in the jar have been found associated with the potential plankton vectors; similar results were obtained with the three substrates (not shown).

In a second series of experiments, we evaluated *V. aestuarianus* capability to interact with marine snow particles and chitin fragments by SEM. To this end, specimens to be examined, consisting of bacteria plus marine snow particles or chitin fragments were prepared as described in Materials and Methods (paragraph 10) and observed at SEM. As shown in Fig.9, bacteria were found adhering to marine snow and chitin particles only in samples containing the substrate inoculated with *V. aestuarianus.*

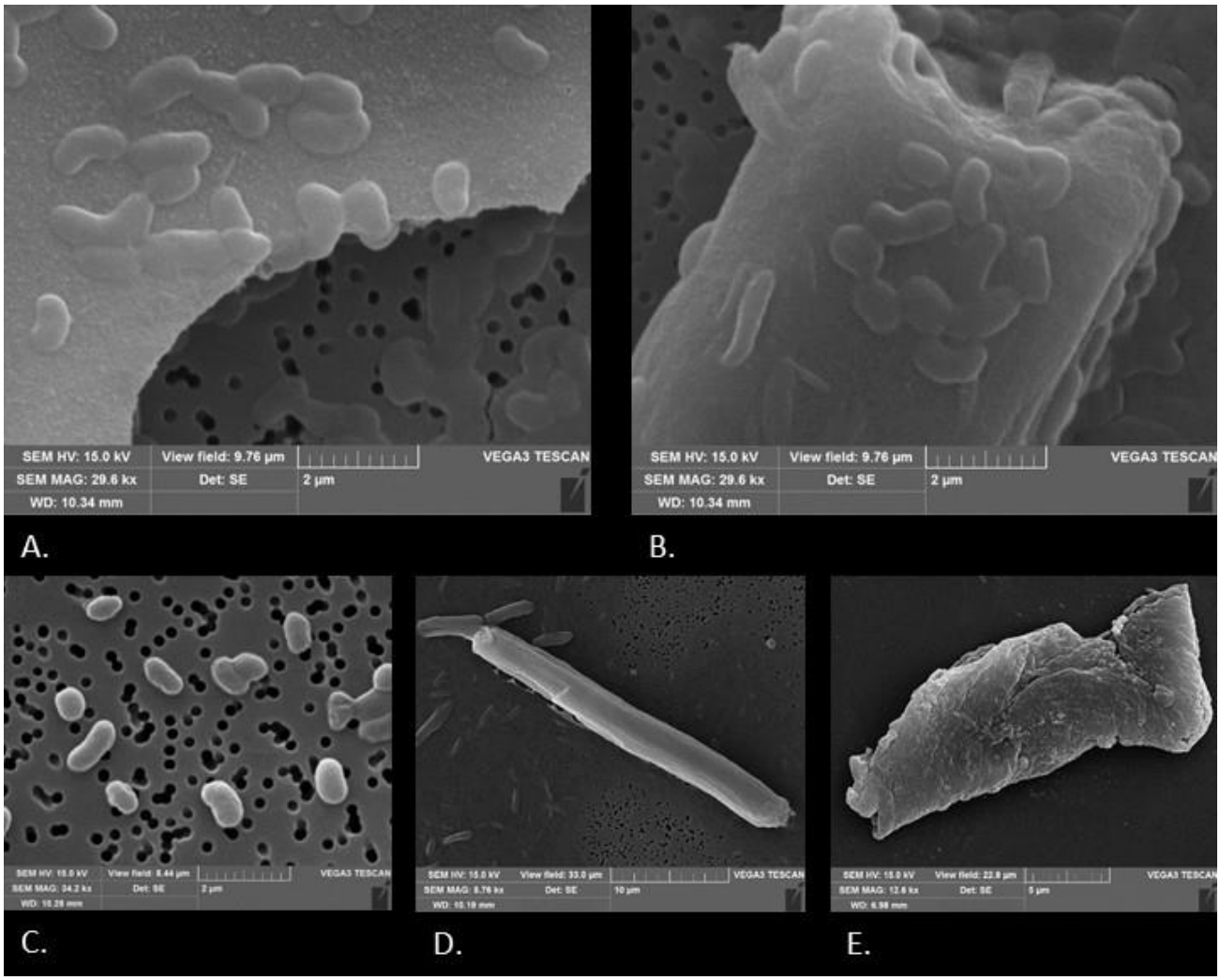

Figure 9. Scanning Electron Microscopy (SEM) of V. aestuarianus 02/041 adhering to plankton substrates. (A) and (B) V. aestuarianus cells on marine snow particles and chitin fragments respectively. (C), (D) and (E) are control sample of, respectively, bacteria, chitin fragments and marine snow particles.

Evaluation of optimal conditions for studying oyster response to exposure by immersion to *V. aestuarianus* bacteria

To evaluate optimal conditions for studying oyster response to exposure by immersion to *V. aestuarianus* bacteria (either plankton associated or non-associated), different experiments were

performed using either “normal” (paragraph 1.2.1) or “stress-exposed” (paragraph 1.2.2) *C. gigas* oysters.

1. In a first series of trials, single oysters were placed into jars containing ASW (salinity 31 ppt) previously inoculated with *V. aestuarianus* 02/041 bacteria alone, or potential vectors (*N. gaditana* cells or marine snow particles or chitin fragments) alone, or mixture of bacteria plus potential vectors, and incubated at 18°C (for details see Materials and Methods). Control oysters in “clean” ASW were also included. Since no mortality was observed in oysters up to 7 days after infection, further experiments were conducted to examine oyster health status following bacterial infection by studying sub-lethal responses. Among biomarkers of oyster health status, hemocyte LMS represents the most sensitive biomarker of stress at the cellular level. Measurements of LMS in animals from climatically and physically diverse ecosystems indicate that it is potentially a universal indicator of health status (OSPAR, 2013). Thus, as above, single oysters were immersed in ASW (salinity 31 ppt) containing *V. aestuarianus* 02/041 bacteria alone, or potential vectors alone, or mixture of bacteria plus potential vectors, and incubated at 18°C (see Materials and Methods). After 2 day incubation, oysters were sampled and hemolymph was extracted to evaluate both *V. aestuarianus* concentration in the hemolymph and hemocyte LMS. No *V. aestuarianus* bacteria were found in the tested hemolymph samples by real-time PCR, and no difference was observed in NRRT between controls and challenged oysters.

2. It is known that oyster susceptibility to infection depends on a number of factors that include pathogen properties, environmental quality and host health status. In particular, mollusks exposed to environmental stressors exhibit higher susceptibility to infection than unexposed ones (Raftos *et al*., 2014; Li *et al*., 2007). For that reason, we evaluated the possibility of using oysters exposed to stress in our infection experiments.

First, we determined the most suitable experimental conditions to efficiently stress oysters without compromising their survival. To this end, we exposed bivalves to temperature or salinity conditions that, although non optimal, do not induce lethal changes (Knowles *et al.,* 2014). Salinity stress was induced by replacing ASW (salinity 31 ppt) with low-salinity ASW (15 ppt or 5 ppt), whereas temperature stress was induced by transferring oysters from 18°C to 30°C or 35°C. The effects of salinity reduction or temperature up-shift on oyster health status were evaluated by analyzing hemocyte LMS after 1 day incubation.

As shown in Table 1, temperature up-shift did not induce marked changes in LMS. In contrast, when salinity was reduced from 31 ppt to 15 ppt and 5 ppt, a decrease in LMS of 38% and 65%, respectively, was observed. These results indicated that, according to international standard for bivalve hemocytes (Martínez-Gómez *et al.* 2015 and 2016*;* Vethaak *et al*., 2015), oyster exposure to 15 ppt low-salinity ASW represents a mild and reversible stress for the bivalve (LMS>50% of control values). Such condition appears to be suitable to induce further stress conditions by infection with *V. aestuarianus* without affecting animal health.

Experimental conditions		NRRT (% of control)
Salinity stress (temperature 18°C)	15ppt	62 ± 11
	5ppt	35 ± 10
Temperaure stress (salinity 31ppt)	30°C	95 ± 9
	35°C	86 ± 10

Table 1. Effects of oyster incubation in either low salinity ASW or high temperature on hemocyte LMS evaluated by the neutral red retention time (NRRT) assay. Data are expressed as percent of control (100%) (salinity stress experiments: 31ppt salinity ASW; temperature stress experiments: 18°C), and represent the mean ± SD of at least three determinations.

As an additional control, we also studied *V. aestuarianus* 02/041 strain long-term survival in 15ppt ASW by evaluating the number of culturable bacteria onto TCBS at timed intervals. As shown in Figure 10 that reports results obtained at different salinity values, bacterial survival during 3 day incubation was approximately the same in ASW 31ppt and 15ppt. In contrast, a reduced survival was observed at lower salinity values.

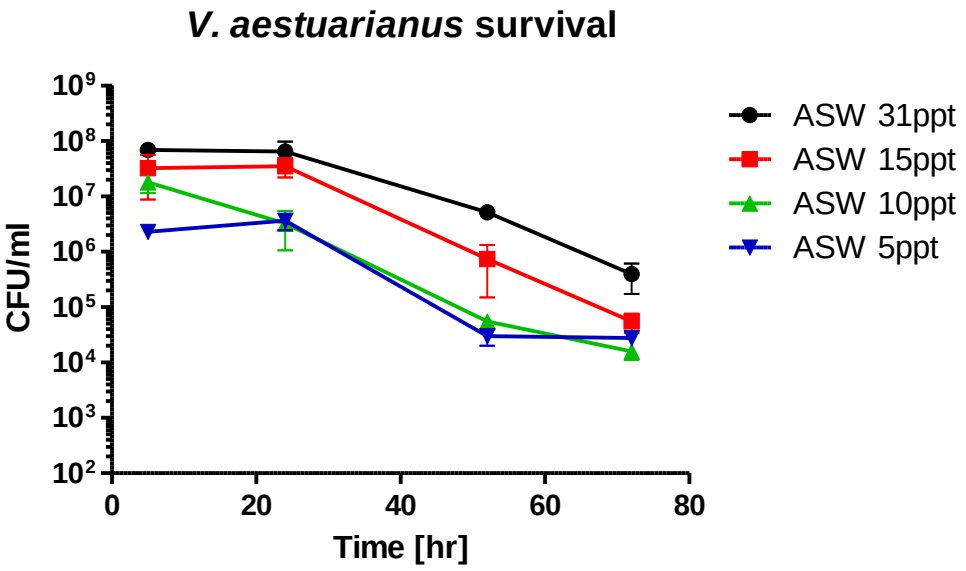

Figure 10. Free-living V. aestuarianus 02/041 survival in different salinities during 3 days

Based on all the above results, further experiments were performed using oysters stressed by incubation in 15 ppt ASW and evaluating oyster health by hemocyte LMS. Experimental design was as reported in Figure 11.

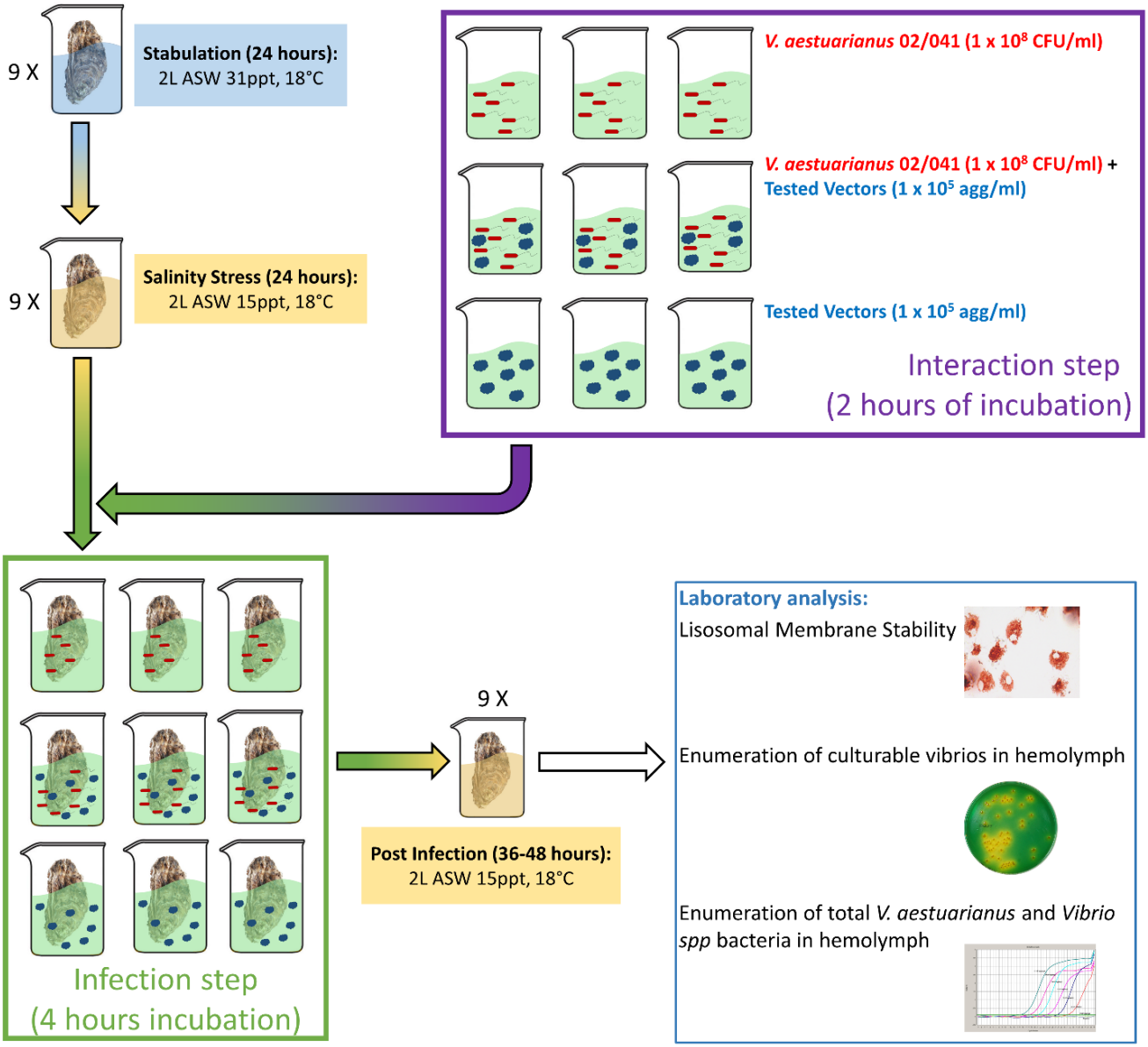

Figure 11. Visual resume of the infection tests. The controls with clean ASW are not represented.

<u>Infection of low-salinity stressed oysters with *V. aesturianus* 02/041 bacteria and *N. gaditana* microalgae</u>

Single low-salinity stressed oysters were added to jars containing ASW (salinity 15ppt) previously inoculated with either *V. aesturianus* 02/041 bacteria alone, or *N. gaditana* cells alone, or mixture of bacteria plus microalgae, and incubated at 18°C (see Figure 11). Control oysters in clean ASW were also included. Two days post infection no oyster mortality was observed; thus, animals were sampled and hemolymph aliquots were analyzed for the presence of culturable and total *V. aesturianus* bacteria and culturable and total vibrios.

As reported in Table 2, that shows the results of a representative experiment, *V. aestuarianus* bacteria were found only in the hemolymph of oysters challenged with bacteria ($5.2x10^3 \pm 7.8x10^2$ bacteria/ml) and, at a twofold higher concentration, in the hemolymph of oysters challenged with bacteria plus *N. gaditana* ($1.2x10^4 \pm 8.1x10^3$ bacteria/ml). Accordingly, a threefold increase in culturable *V. aestuarianus* bacteria was observed in samples infected with bacteria plus microalgae in comparison to those infected with bacteria alone ($6.1x10^3 \pm 8.3x10^2$ CFU/ml *vs*

$2.1x10^3 \pm 9.2x10^2$ CFU/ml). It is noteworthy that about half of *V. aestuarianus* 02/041 bacteria in the hemolymph was culturable. As regards total vibrios and culturable vibrio-like bacteria (Table 2), their concentration in the hemolymph of oysters challenged with *N. gaditana* only ($2.7x10^4 \pm 5.4x10^3$ bacteria/ml and $9.6x10^3 \pm 9.4x10^2$ CFU/ml) was similar to that of unchallenged control ($1.5x10^4 \pm 3.4x10^3$ bacteria/ml and $8x10^3 \pm 6.6x10^2$ CFU/ml). In oysters challenged with bacteria only or bacteria plus microalgae, vibrio-like bacteria concentrations increased to $3.5x10^4 \pm 6.3x10^3$ bacteria/ml and $1.5x10^4 \pm 4.3x10^3$ CFU/ml and to $8.4x10^4 \pm 7.2x10^3$ bacteria/ml and $3.2x10^4 \pm 5.1x10^3$ CFU/ml, respectively.

Analysis of hemocyte LMS, utilized as a biomarker of oyster health status, showed that NRRT in oysters challenged with bacteria and microalgae was significantly lower that NRRT in samples challenged with either bacteria alone or algae alone (Table 3).

	V. aestuarianus [bacteria/ml]	*V. aestuarianus* [CFU/ml]	*Vibrio* spp. [bacteria/ml]	*Vibrio*- like [CFU/ml]
ASW	0	0	$1.5 \times 10^4 \pm 3.4 \times 10^3$	$8 \times 10^3 \pm 6.6 \times 10^2$
N. gaditana	0	0	$2.7 \times 10^4 \pm 5.4 \times 10^3$	$9.6 \times 10^3 \pm 9.4 \times 10^2$
V. aestuarianus	$5.2 \times 10^3 \pm 7.8 \times 10^2$	$2.1 \times 10^3 \pm 9.2 \times 10^2$	$3.5 \times 10^4 \pm 6.3 \times 10^3$	$1.5 \times 10^4 \pm 4.3 \times 10^3$
N. gaditana + *V. aestuarianus*	$1.2 \times 10^4 \pm 8.1 \times 10^3$	$6.1 \times 10^3 \pm 8.3 \times 10^2$	$8.4 \times 10^4 \pm 7.2 \times 10^3$	$3.2 \times 10^4 \pm 5.1 \times 10^3$

Table 2. V. aesturianus 02/041 and Vibrio-like bacteria concentration in hemolymph of oysters infected with either V. aesturianus 02/041 bacteria alone, or N. gaditana cells alone, or mixture of bacteria plus microalgae (reported values refer to a single representative experiment and are the mean of at least three determinations ± standard deviation). Similar results were obtained in other triplicate experiments.

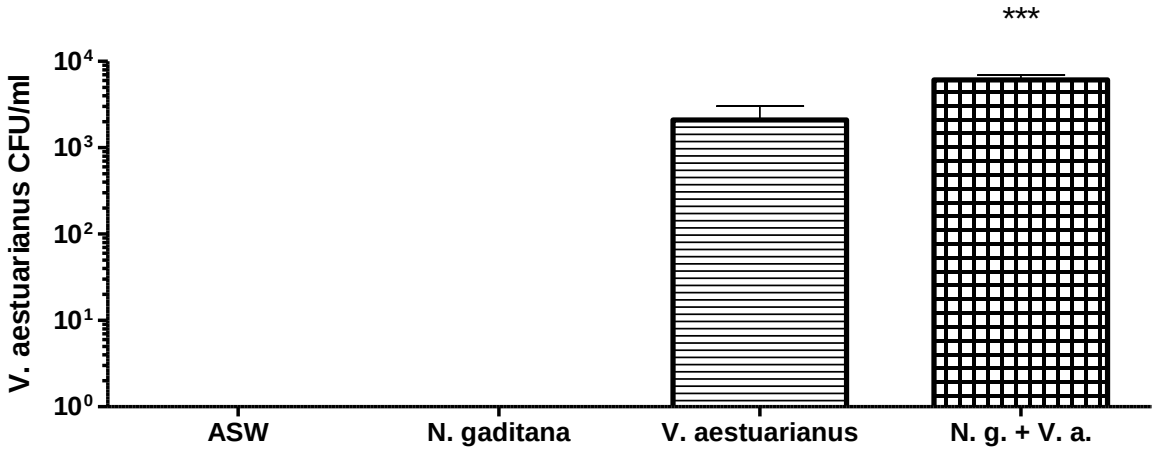

Figure 12. Culturable V. aestuarianus 02/041 concentration hemolymph of infected oysters expressed as bacteria x ml-1. The oysters infected with the mixture of bacteria and N. gaditana showed a statistically significant increase (Bonferroni: p<0.001)

	NRRT (% of control)	NRRT decrease (% of control)
N. gaditana	102 ± 3	+2% ± 3
V. aestuarianus	99 ± 8	-1% ± 8
N. gaditana + V. aestuarianus	50 ± 8	-50% ± 8

Table 3. Effects of low salinity stressed oyster challenge with either V. aesturianus 02/041 bacteria alone, or N. gaditana cells alone, or mixture of bacteria plus microalgae on hemocyte LMS evaluated by the neutral red retention time (NRRT) assay (Data are expressed as percent of control values (uninfected control, 100%), and represent the mean ± SD of at least three determinations)

Infection of low-salinity stressed oysters with *V. aesturianus* 02/041 and Marine snow

Single low-salinity stressed oysters were added to jars containing ASW (salinity 15ppt) previously inoculated with either *V. aesturianus* 02/041 bacteria alone, or marine snow particles alone, or mixture of bacteria plus marine snow, and incubated at 18°C (see Materials and Methods). Control oysters in clean ASW were also included. Two days post infection no oyster mortality was observed, animals were sampled and hemolymph aliquots were analyzed for the presence of total and culturable *V. aesturianus* cells and total and culturable vibrios.

As reported in Table 4, that shows the results of a representative experiment, *V. aestuarianus* bacteria were found only in the hemolymph of oysters challenged with bacteria ($4.1x10^3 \pm 2.3x10^2$ bacteria/ml) and, at an higher concentration, in the hemolymph of oysters challenged with bacteria plus marine snow ($6.2x10^4 \pm 4.1x10^3$ bacteria/ml). Accordingly, an increase in culturable *V. aestuarianus* bacteria was observed in samples infected with bacteria plus marine snow in comparison to those infected with bacteria alone ($2.4x10^3 \pm 3.2x10^2$ CFU/ml *vs* $1.8x10^3 \pm 5.3x10^2$ CFU/ml). As for the experiment conducted with microalgae, an high proportion of *V. aestuarianus* 02/041 bacteria in the hemolymph was culturable. As regards total vibrios and culturable vibrio-like bacteria (Table 4), their concentration in the hemolymph of oysters challenged with marine snow only ($1.1x10^4 \pm 8.4x10^2$ bacteria/ml and $8.8x10^3 \pm 7.3x10^2$ CFU/ml) was similar to that of uninfected control ($1.4x10^4 \pm 6.6x10^3$ bacteria/ml and $9.2x10^3 \pm 4.7x10^2$ CFU/ml). In oysters challenged with bacteria only or bacteria plus marine snow, vibrio-like bacteria concentrations increased to $2.6x10^4 \pm 3.3x10^3$ bacteria/ml and $7.8x10^4 \pm 2.9x10^3$ CFU/ml and to $3.1x10^4 \pm 6.4x10^3$ bacteria/ml and $2.2x10^4 \pm 1.9x10^3$ CFU/ml, respectively.

Analysis of hemocyte LMS, utilized as a biomarker of the oyster health status, showed that NRRT in oysters challenged with bacteria and marine snow was lower that NRRT in samples challenged with either bacteria alone or marine alone (Table 5).

	V. aestuarianus [bacteria/ml]	*V. aestuarianus* [CFU/ml]	*Vibrio* spp. [bacteria/ml]	*Vibrio*- like [CFU/ml]
ASW	0	0	$1.4 \times 10^4 \pm 6.6 \times 10^3$	$9.2 \times 10^3 \pm 4.7 \times 10^2$
Marine Snow	0	0	$1.1 \times 10^4 \pm 8.4 \times 10^3$	$8.8 \times 10^3 \pm 7.3 \times 10^2$
V. aestuarianus	$4.1 \times 10^3 \pm 2.3 \times 10^2$	$1.8 \times 10^3 \pm 5.3 \times 10^2$	$2.6 \times 10^4 \pm 3.3 \times 10^3$	$7.8 \times 10^4 \pm 2.9 \times 10^3$
Marine Snow + *V. aestuarianus*	$6.2 \times 10^4 \pm 4.1 \times 10^3$	$2.4 \times 10^3 \pm 3.2 \times 10^2$	$3.1 \times 10^4 \pm 6.4 \times 10^3$	$2.2 \times 10^4 \pm 1.9 \times 10^3$

Table 4. V. aestuarianus 02/041 and Vibrio-like bacteria concentration in hemolymph of oysters infected with either V. aestuarianus 02/041 bacteria alone, or marine snow alone, or mixture of bacteria plus marine snow. Reported values refer to a single representative experiment and are the mean of at least three determinations ± standard deviation. Similar results were obtained in other triplicate experiments. Differences between samples infected with bacteria only and samples infected with bacteria plus microalgae are statistically significant ($p < 0.05$).

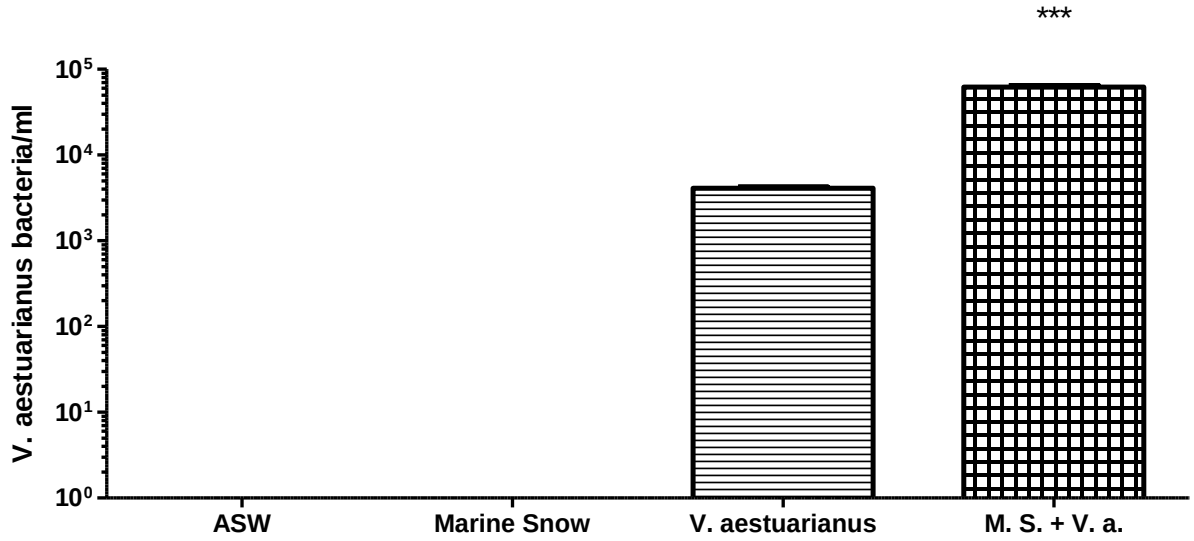

Figure 13. V. aestuarianus 02/041 concentration hemolymph of infected oysters expressed as bacteria x ml-1. The oysters infected with the mixture of bacteria and Marine Snow showed a statistically significant increase (Bonferroni: $p<0.001$)

	NRRT (% of control)	**NRRT decrease (% of control)**
Marine Snow	108 ± 2	+8% ± 2
V. aestuarianus	90 ± 7	-10% ± 7
Marine Snow + *V. aestuarianus*	70 ± 6	-30% ± 6

Table 5. Effects of low salinity stressed oyster challenge with either V. aesturianus 02/041 bacteria alone, or marine snow alone, or mixture of bacteria plus marine snow on hemocyte LMS evaluated by the neutral red retention time (NRRT) assay (Data are expressed as percent of control values (uninfected control), and represent the mean ± SD of four experiments in triplicate)

Infection of low-salinity stressed oysters with *V. aesturianus* 02/041 and chitin

Single low-salinity stressed oysters were added to jars containing ASW (salinity 15 ppt) previously inoculated with either *V. aesturianus* 02/041 bacteria alone, or chitin fragments alone, or mixture of bacteria plus chitin, and incubated at 18°C (see Materials and Methods). Control oysters in clean ASW were also included. As above, two days post infection no oyster mortality was observed; then, animals were sampled and hemolymph aliquots were analyzed for the presence of total *V. aesturianus* cells and culturable and total vibrios.

As shown in Table 6, *V. aestuarianus* bacteria were found only in the hemolymph of oysters challenged with bacteria ($1.9x10^3 \pm 7.6x10^2$ bacteria/ml and $7.4x10^2 \pm 9.2x10^1$ CFU/ml) and in the hemolymph of oysters challanged with bacteria plus chitin ($1.1x10^3 \pm 8.1x10^2$ bacteria/ml and $5.3x10^2 \pm 8.4x10^1$ CFU/ml). As regards total vibrios and culturable vibrio-like bacteria, their concentration in hemolymph of oysters challenged with chitin only was similar to that of unchallenged control ($3.3x10^4 \pm 9{,}1x10^3$ bacteria/ml and $5.1x10^3 \pm 9.7x10^2$ CFU/ml); in oysters challenged with bacteria only or bacteria plus chitin, concentrations reached values of $7.1x10^4 \pm 8.9x10^3$ bacteria/ml and $2{,}1x10^4 \pm 3.4x10^3$ CFU/ml and to $5.2x10^4 \pm 8.2x10^3$ bacteria/ml and $1.1x10^4 \pm 8.9x10^3$ CFU/ml, respectively.

Analysis of hemocyte LMS of the tested samples (treated and untreated with bacteria and chitin both alone and in combination) showed no statistically significant differences in NRRT (not shown).

	V. aestuarianus [bacteria/ml]	*V. aestuarianus* [CFU/ml]	*Vibrio* spp. [bacteria/ml]	*Vibrio*- like [CFU/ml]
ASW	0	0	$2.1 \times 10^4 \pm 7.2 \times 10^3$	$3.1 \times 10^3 \pm 8.4 \times 10^2$
Chitin particles	0	0	$3.3 \times 10^4 \pm 9.1 \times 10^3$	$5.1 \times 10^3 \pm 9.7 \times 10^2$
V. aestuarianus	$1.9 \times 10^3 \pm 7.6 \times 10^2$	$7.4 \times 10^2 \pm 9.2 \times 10^1$	$7.1 \times 10^4 \pm 8.9 \times 10^3$	$2.1 \times 10^4 \pm 3.4 \times 10^3$
Chitin particles + *V. aestuarianus*	$1.1 \times 10^3 \pm 8.1 \times 10^2$	$5.3 \times 10^2 \pm 8.4 \times 10^1$	$2.1 \times 10^4 \pm 7.2 \times 10^3$	$3.1 \times 10^4 \pm 8.4 \times 10^3$

Table 6. V. aesturianus 02/041 and Vibrio-like bacteria concentration in hemolymph of oysters infected with either V. aesturianus 02/041 bacteria alone, or chitin fragments alone, or mixture of bacteria plus chitin (reported values refer to a single representative experiment and are the mean of at least three determinations ± standard deviation. Similar results were obtained in other triplicate experiments.

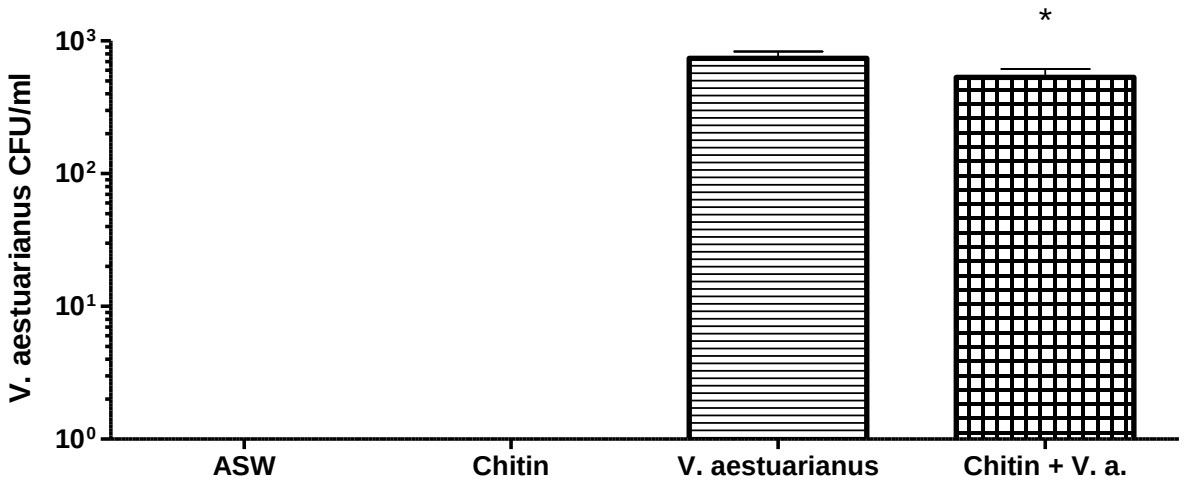

Figure 14. Culturable V. aestuarianus 02/041 concentration hemolymph of infected oysters expressed as bacteria x ml-1. The oysters infected with the mixture of bacteria and chitin particles showed a statistically significant decrease (Bonferroni: p<0.05)

Vibrio interaction with plankton substrates and aminopeptidase production

Experiments were conducted to study if interactions of *V. aestuarianus* 02/041, *V. tasmaniensis* LGP32, *V. coralliilyticus* ATCC BAA450, *V. harveyi* VH2 and *V. tapetis* CECT 4600 strains with marine snow particles and chitin fragments might influence the production of extracellular virulence-related products. To this end, aminopeptidase activity of the strains was measured through fluorogenic assays in the presence or absence of the different substrates.

V. aestuarianus 02/041

Extracellular aminopeptidase (L-leucine-4-methylcoumarinyl-7-amide, Leu-MCA) activity measured for *V. aestuarianus* 02/041 in ASW (salinity 15 ppt) showed average values of 24.6±0.7 µmol/10^8cells/h (Table 1). In the presence of Marine snow particles, aminopeptidase activity increased up to 37.0±0.2 µmol/10^8cells/h representing a 54 % increase if compared with ASW values. In contrast, in the presence of chitin fragments no increase was observed for bacterial protease activity (22.6±0.2 µmol/10^8cells/h).

V. tasmaniensis LGP32

Extracellular aminopeptidase activity measured for *V. tasmaniensis* LGP32 in ASW (salinity 15 ppt) showed average values of 7.1±0.2 µmol/10^8cells/h (Table 1). No increase of aminopeptidase activity was observed either in the presence of Marine snow particles (7.0±0.2 µmol/10^8cells/h) or chitin fragments (6.6±0.2 µmol/10^8cells/h).

V. coralliilyticus ATCC BAA450

Extracellular aminopeptidase activity measured for *V. coralliilyticus* ATCC BAA450 in ASW (salinity 15 ppt) showed average values of 26.8±0.9 µmol/10^8cells/h (Table 1). In the presence of Marine snow particles, aminopeptidase activity increased up to 42.4±0.8 µmol/10^8cells/h representing a

58 % increase if compared with ASW values. In contrast, no significant increase was observed for bacterial aminopeptidase activity in the presence of chitin fragments (28.2±o.8 µmol/10^8cells/h).

V. harveyi VH2

Extracellular aminopeptidase activity measured for *V. harveyi* VH2 in ASW (salinity 15 ppt) showed average values of 24.7±0.6 µmol/10^8cells/h (Table 1). In the presence of Marine snow particles, aminopeptidase activity increased up to 37.8±0.4 µmol/10^8cells/h representing a 53 % increase if compared with ASW values. In contrast, no significant increase was observed for bacterial aminopeptidase activity in the presence of chitin fragments (21.9±0.8 µmol/10^8cells/h).

V. tapetis CECT 4600

Extracellular aminopeptidase activity measured for *V. tapetis* CECT 4600 in ASW (salinity 15 ppt) showed average values of 23.6±0.4 µmol/10^8cells/h (Table 1). No significant increase of aminopeptidase activity was observed either in the presence of Marine snow particles (21.6±0.8 µmol/10^8cells/h) or chitin fragments (21.7±0.4 µmol/10^8cells/h).

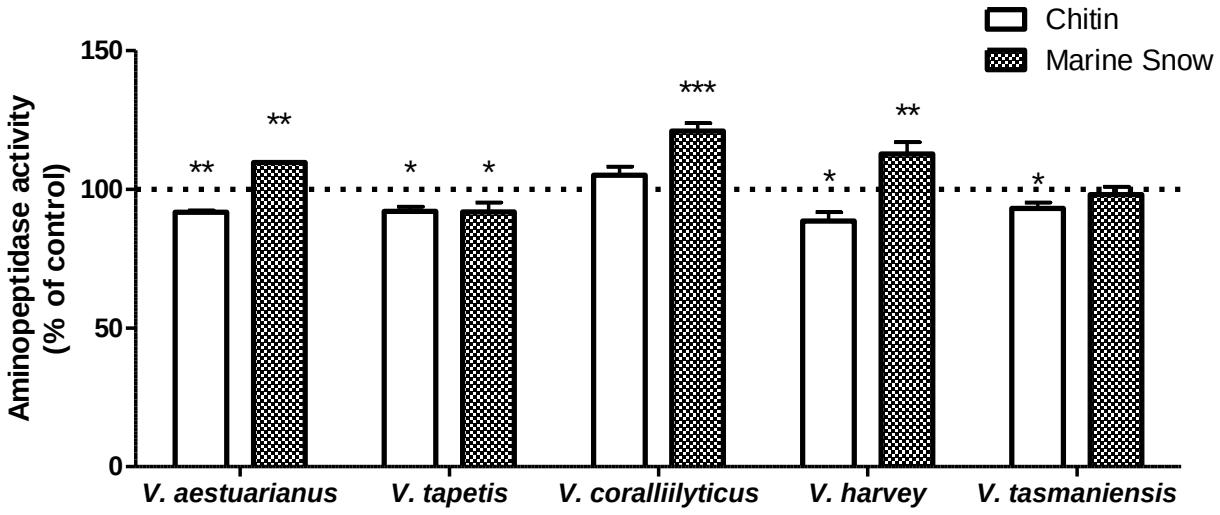

Figure 15. Aminopeptidase activity compared to control of 5 vibrios species in presence of Marine Snow or Chitin particles. Data are expressed as percent of control (100%) (bacteria with ASW), and represent the mean ± SD of at least three determinations.

Experimental conditions	*V. aestuarianus* 02/041	*V. tasmaniensis* LGP32	V. *coralliilyticus* ATCC BAA450	*V. harveyi* VH2	*V. tapetis* CECT 4600
ASW (control)	24.6 ± 0.7	7.1 ± 0.2	26.8 ± 0.9	24.7 ± 0.6	23.6 ± 0.4
ASW + chitin	22.6 ± 0.2	6.6 ± 0.2	28.2 ± 0.8	21.9 ± 0.8	21.7 ± 0.4
ASW + marine snow	27.0 ± 0.2	7.0 ± 0.2	32.4 ± 0.8	27.8 ± 0.4	21.6 ± 0.8

Table 7. Effect of the presence of either chitin fragments or marine snow particles on extracellular aminopeptidase activity (µmol/10^8 cells/h) produced by different Vibrio strains.

Discussion

Some *Vibrio* spp. are able to interact with environmental chitinous substrates (including plankton crustaceans) using different adhesion ligands (Vezzulli *et al.,* 2016). This capability makes plankton organisms a potential environmental reservoir of Vibrios pathogenic for both humans (*e.g*., *V. cholerae*) and animals (*e.g*., *V. aestuarianus*). In addition, some plankton species have been associated to the transmission of *V. cholerae* to humans (Colwell *et al*., 2003, Rawlings *et al*., 2007). In this context, this work aimed to determine whether plankton may also constitute an important driver of pathogen transmission to bivalve hosts (*i.e.*, oysters).

Three representative marine substrates were evaluated as potential target of *V. aestuarianus* 02/041 strain, one of the most virulent pathogen of *C. gigas*: *N. gaditana* cells, marine snow particles and chitin fragments.

First, we confirmed that the strain is able to adhere to all the tested substrates. Then, based on results from artificial infection experiments, we showed that phytoplankton cells (*N. gaditana*) and marine snow particles significantly promote *V. aestuarianus* 02/041 intake by *C. gigas* maintained under stressful conditions in the laboratory. Such intake is associated with a decrease in Lysosomal membrane stability of oyster hemocyte indicating a compromised health status of infected oysters. In contrast, chitin particles did not appear to favor pathogen transmission to the bivalve host. Phytoplankton cells and marine snow particles both constitute a food source for *C. gigas* and can be filtered efficiently by the bivalve filtration system (*e.g.* due to optimal size and chemical properties). Pathogenic bacteria such as *V. aestuarianus* 02/041 might take advantage of this by adhering to food particles and entering the bivalve host more efficiently. Bacteria attached to environmental substrates could also be less sensitive to bivalve antimicrobial activity (as reported for *Vibrio* species involved in human infections), although, at present, this interpretation remains speculative.

Other laboratory experiments were performed to study if interactions of *V. aestuarianus*, *V. tasmaniensis*, *V. coralliilyticus*, *V. harveyi* and *V. tapetis* strains with marine snow particles and chitin fragments might directly/indirectly influence production of extracellular virulence-related products (*i.e*., protease). Results from these experiments suggest that for some *Vibrio* strains, the interaction with plankton substrates might directly influence bacterial virulence by modulating extra-cellular protease activity. In particular, interaction with marine snow particles significantly increased aminopeptidase activity rates in *V. aestuarianus* 02/041, *V. coralliilyticus* ATCC BAA450 and *V. harveyi* VH2. In contrast, this was not observed in *V. tasmaniensis* LGP32 and *V. tapetis* CECT 4600. Similarly, bacterial interaction with chitin fragments did not have an effect on protease activity rates whatever the tested strain was.

In conclusion, results from these studies not only support the role of plankton as an environmental reservoir for pathogenic bacteria involved in *C. gigas* mortalities, but also suggest that marine snow and plankton organisms (especially phytoplankton) might represent suitable vehicle for microbial pathogens and favor their transmission to the bivalve host. Interestingly, the presence of marine snow particles had also an effect on aminopeptidase produced by bacteria by either increasing its synthesis or improving its activity. Considering that *Vam* metalloprotease is one of the most important *V. aestuarianus* virulence factor, this might greatly affect virulence of the *V. aestuarianus* 02/041 bacteria.

Pathobiota associated at mortality events of Pacific Oyster

Objectives

In Europe mass mortality episodes of the *C. gigas* in farming areas were reported at increasing frequency in recent years and are attributed to complex interactions among oysters, microbial pathogens and environmental variables (Pernet *et al.,* 2012). In particular, stressful environmental conditions such as warmer seawater temperatures were observed to favour shift of *C. gigas* bacterial communities toward pathogen-dominated communities also promoting colonization by secondary opportunistic pathogens and non-resident microbial species (Lokmer and Wegner, 2015).

Although specific microbial pathogens (*e.g*. OsHV-1 and *V. aestuarianus*) and some virulence factors have been identified to play a role in oyster diseases (Travers *et al.,* 2015) there is an emerging view that microbial infections may derive from the contribution of different microbial species/strains that act as a "community of pathogens" rather than a single species/strain as the only etiological agent (Lemire *et al.,* 2015). Under this perspective, evidence has been provided supporting the view that oyster infections might be seen as infectious disorders caused by the contribution of a larger number of pathogens (*e.g*. populations or consortia) than previously thought (Lemire *et al.,* 2015). The question now is no longer whether microorganisms are involved in the pathogenesis of such diseases, but which specific microbial species or strains are involved.

To understand this aspect, *C. gigas* samples were selected to study all the pathogenic component of the microbiota, the pathobiota. An innovative method, the target enrichment were applied to the samples to enrich a selection of targets custom selected comprising phylogenetic markers and virulence factors of most interesting pathogens.

These Pacific Oyster samples were selected between 462 oyster samples collected from two European farming areas: delta Ebro river (Spain) and Dungarvan bay (Ireland), during mortalities events of adults and juveniles. To evaluate the presence of the pathogens in the surrounding environment, also other elements collected close to Dungarvan bay aquaculture were screened: bivalves (*Littorina littorea*, clam, cockles) and other environmental matrices (water and sediment).

The samples were screened for main *C. gigas* bacterial pathogens (*V. aestuarianus*) presence and other Vibrios associated to mortality outbreaks (*V. splendidus* clade and *V. coralliilyticus*). These results were merged with other analysis performed from project partners (*e.g.* OsHV-1 µvar, mortality rate during outbreaks) to select samples most interesting and representative for apply target enrichment.

Samples collection during mortality episodes in Europe

In the frame of the EU funded H2020 project VIVALDI (Preventing and mitigating farmed bivalve diseases) 462 *C. gigas* samples were collected during 13 sampling campaigns conducted by project partners from March 2016 to October 2017 at different European sites (Ebro delta, Spain and Dungarvan bay, Ireland) experiencing mass mortality episodes during the summer periods (Figure 16). The local partners collected the *C. gigas* samples and extracted the DNA by following these steps: immediately after collection samples were transported to the laboratory and bivalve tissues were extracted from single specimen, gently washed with 10ml of ASW and homogenized in a Polytron PT 3000 Kinematica AG homogenizer (K. H. Brinkmann GmbH & Co. KG, Werdohl, Germany). 0.25 g of oysters homogenates was then used for DNA extraction with High Pure Polymerase Chain Reaction (PCR) Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Figure 16. C. gigas aquaculture sampling site.

Other samples were collected from Dungarvan bay during outbreaks: other bivalves (27 *Littorina littorea*, 5 clams and 1 cockle), 14 sediments and 3 marine water. Bivalves were processed as oysters samples: moved to the laboratory, washed with sterile ASW, homogenized in a Polytron and the homogenates used for DNA extraction with High Pure Polymerase Chain Reaction (PCR) Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

0.22±2 g of sediments samples were used to DNA extraction with PowerSoil DNA Isolation Kit (MO BIO Laboratories, USA) according to the manufacturer's instructions.

1 liter of marine water samples were filtered through nucleopore-filters (45 mm diameter, 0.2 µm pore size, Track-etch, Whatman Corp) and DNA extraction was extracted by PowerWater DNA Isolation Kit Sample (MO BIO Laboratories, USA) according to the manufacturer's instructions.

Potential Vibrio spp. pathogen screening by real-time PCR

All *C. gigas* samples were screened for the presence of OsHV-1 and *Vibrio aestuarianus* by real-time PCR as previously described (Webb *et al.,* 2007; IFREMER, 2013). The partners who sampled *C. gigas* did the screening for OsHV-1 presence.

Some of the *C. gigas* samples were screened also for *Splendidus* clade spp. by real-time PCR as previously described (IFREMER, 2013).

Other samples (bivalves, sediment and filtered water) were screened initially for Vibrios presence and then for the presence of pathogens *V. aestuarianus*, *Splendidus* clade and *V. coralliilyticus*.

For detection of *V. splendidus* clade spp. was used a TaqMan real-time PCR protocol with the LightCycler (Roche Diagnostic). *V. splendidus* clade spp. primers and probe (SPR2 5'-CAATGGTTATCCCCCACATC-3' and SPF2 5'-ATCATGGCTCAGATTGAACG-3' and 16S probe 5'-6FAM-CCCATTAACGCACCCGAAGGATTG-BHQ1-3') were used in the assays. Each reaction mixture contained 1X LightCycler TaqMan Mastermix (Roche Diagnostics) and 1 µM of each primer and 0.1 µM of each probe in a final volume of 20 µl. The PCR program used was as follows: initial denaturation at 95°C for 10 min, subsequent 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s and elongation at 72°C for 1 s, followed by final elongation at 72°C for 10 min.

For detection of *V. coralliilyticus,* was using a SYBR Green real-time PCR protocol with the LightCycler (Roche Diagnostic). Primers for *V. coralliilyticus* zinc-metalloprotease (VcpARTF 5'-AGCTACGACTGCCGCCCTTAC-3' and VcpARTR 5'-GGAGCCCTTTCACTTACGATGTTG-3') were used in the assay. Each reaction mixture contained 1X LightCycler SYBR Green Mastermix (Roche Diagnostics) and 1 µM of each primer in a final volume of 20 µl. The PCR program used was as follows: initial denaturation at 95°C for 10 min, subsequent 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 5 s and elongation at 72°C for 4 s, followed by final elongation at 72°C for 30 s.

The protocols for *V. aestuarianus* and Vibrios detection are illustrated in the previous chapter.

For all the real-time PCR were added five microliters of DNA template to the reaction mixture.

Accurately quantified copy number genomic DNA of *V. tasmaniensis* LPG32, *V. aestuarianus* 01/32 and *V. coralliilyticus* ATCC BAA 450 strains were used as a standard.

Analysis of Pacific Oyster "microbiota" by 16S rRNA gene-based profiling of the bacterial community

16SrDNA PCR amplicon libraries were generated from genomic DNA extracted from *C. gigas* samples using primers amplifying the V4 region of the 16S rRNA gene of bacteria. All primers were custom designed to include 16SrRNA complementary regions plus the complementary sequences to the Ion Torrent specific adapters (Table 8). Two PCR assays were performed. A 1st target enrichment PCR assay with the 16S conserved primers. A 2nd PCR assay, with customized primers and included adapters' complementary regions. The obtained libraries were sequenced using an Ion Torrent (PGM) Platform.

First PCR	
515f_UNI1+ *Unitail 1*	*CAG GAC CAG GGT ACG GTG* GTG CCA GCM GCC GCG GTA A
Second PCR	
802 R + *Unitail 2*	*CGC AGA GAG GCT CCG TG* T ACN VGG GTA TCT AAT CC
806 R + *Unitail 2*	*CGC AGA GAG GCT CCG TG* G ACT ACH VGG GTW TCT ATT

Table 8. Primers sequences for the 16S rRNA gene-based microbiome profiling.

Bioinformatics analysis of NGS data was performed using the Microbial Genomics module (version 1.3) work-flow of the CLC Genomics Workbench (version 9.5.1) and other comparable software. After quality trimming based on quality scores and length trimming, reads were clustered at 97% level of similarity into Operational Taxonomical Units (OTUs). Chimera detection and removal were performed. Ribosomal RNA gene reads were classified against the non-redundant version of the SILVA SSU reference taxonomy (release 119; http://www.arb-silva.de). Alpha diversity analysis was then conducted on total OTUs by constructing rarefaction curves calculated by sub-sampling OTUs abundances in the different samples at different depths. Beta diversity analysis was also performed by calculating Bray-Curtis distances between each pair of samples and applying Principal Coordinate Analysis (PCoA) on the distance matrices.

The core microbiota, defined as the microbial taxa belonging to OTUs present in all the samples, was analyzed using the Corbata software (CORe microbiome Analysis Tools, Li *et al.,* 2013). A two-parameter model (Ubiquity-Abundance) was applied to quantitatively identify the core taxonomic members of each sample group microbiota considering the different conditions. This software allows the determination of the core members (high abundance and high ubiquity) of a single set of samples, but also allows the comparison of the core microbiota of two different groups of samples. Furthermore, the analysis of the "minor" core can be performed, thus, identifying bacterial groups at a very low abundance but high ubiquity. The AWKS statistic test (Abundance-Weighted Kolmogorov-Smirnov) was used to compared core microbiota profiles among samples.

Target Enrichment for the analysis of the *C. gigas* Pathobiota

Target enrichment and next-generation sequencing protocol for the analysis of the bivalve "pathobiota" was developed following the approach previously applied for the target sequencing of *V. cholerae* DNA in complex environmental samples (Vezzulli *et al.*, 2017).

To this aim 884 *Vibrio* phylogenetic and virulence markers as well as other bivalve and marine invertebrates microbial pathogen markers (Travers *et al.,* 2015, Rosenberg *et al.,* 2007) with average length of ca 400nt were identified and used to produced 100-mer biotinylated RNA baits for selective capturing of target DNA markers via hybridization. Baits were produced using the MYcroarray target enrichment proprietary technology (MYcroarray, Ann Arbor, MI, USA). 500ng of total baits was used for capture and was capable of enriching single-copy nuclear loci i.e. >99.5% of the capture target region.

Genomic DNA extracted from bivalve samples was sized on an Agilent Bioanalyzer and enzymatically fragmented using the KAPA Frag Kit (Roche Diagnostics, Mannheim, Germany) protocol to an average size of about 600bp. The fragmented DNA was used for the production of an indexed library for next-generation sequencing on the Illumina platform (Illumina, Inc) using the KAPA HyperPlus Kit for Illumina (Roche Diagnostics, Mannheim, Germany). About 200 ng of

the produced library was used for target DNA capturing using the MYbaits protocol (MYcroarray, Ann Arbor, MI, USA) following the manufacture instructions.
The DNA library was heat-denatured and hybridized to the RNA baits in stringent conditions. After hybridization, the biotinylated baits hybridized to captured material were pulled out of the solution with streptavidin-coated magnetic beads and the captured genomic DNA was released by chemical degradation of the RNA baits. Post-capture PCR amplification was finally carried out.
In addition to bivalve samples, DNA extracted from a Mock community sample (positive control) composed of equal amount of genomic DNA from *V. cholerae* O139 5424, *V. tasmaniensis* LGP 32, *Vibrio alginolyticus, V. cholerae* non O1/O139, *Vibrio mimicus* CP192, *Vibrio cholerae* O1 classico CD81, *Vibrio aestuarianus* 01/032, *V. cholerae* N16961 El Tor, *Vibrio coralliilyticus* ATCC BAA 450, *Vibrio tapetis* CECT 4600, *Vibrio vulnificus* ATCC 275262, *V. parahaemolyticus* 54496, *Escherichia coli* ATCC 2922, *Serratia marcescens, Enterococcus faecalis* ATCC 29212. A sterile seawater sample (negative control) was also analyzed following the same protocol.
All samples libraries were then pooled and sequenced on a MiSeq Illumina™ platform (V3 flow cell, 600 cycles, 25M reads 250bp pair ends). After quality trimming, sequence reads were mapped against reference sequences of phylogenetic and virulence markers used to produced the baits using the CLC mapping tool set with length fraction of 0.5 and similarity fraction of 0.95. Consensus sequences were then produced from output with a minimum of 10 reads mapping on the reference sequences and blasted against the nucleotide collection (nr) database of NCBI for classification.

Results

Pathogens screening results

A total of 462 *C. gigas* samples collected from two VIVALDI sampling sites (Ebro delta and Dungarvan bay) were screened for the presence of OsHV-1 and *V aestuarianus* using quantitative real-time PCR (Table 9). Samples collected during mortality episodes scored positive for at least one of the two pathogens suggesting a strong link between presence of these microorganisms in the oyster tissues and development of the disease. In particular, *V. aestuarianus* was found associated to adult *C. gigas* mortality observed in the Ebro delta on 13th Apr 2016 (up to 50% mortality) and 31th May 2017 (85% mortality) and in Dungarvan bay on 3rd October 2016 (20% mortality) (Table 9). OsHV-1 was found associated to spat/juvenile *C. gigas* mortality in at least 12 mortality episodes observed in the Ebro delta and Dungarvan bay from 2016 to 2017 with mortality ranging from 20% to 100% (Table 9).

Table 9. Summary of screening results of C. gigas samples collected from Ebro delta and Dungarvan bay.

Dungarvan Bay (Ireland)				
Date	**VIVALDI Code**	**Mortality**	**Results OsHV-1**	**Results *V. aestuarianus***
05/07/2016	**MI-2016-001 (Adult)**	20%	0+/30	2+/30
05/07/2016	**MI-2016-001 (Spat)**	70-100%	25+/30	3+/30
03/10/2016	**MI-2016-002 (Adult)**	end of mortality	0+/30	11+/30
03/10/2016	**MI-2016-002 (Spat)**	end of mortality	12+/30	1+/30
10/01/2017	**MI-2017-001 (Adult)**	no mortality	0+/30	0+/30
10/01/2017	**MI-2017-001 (Spat)**	no mortality	0+/30	0+/30

Ebro delta (Spain)					
Date	**VIVALDI Code**	**Mortality**	**Results OsHV-1**	**Results *V. aestuarianus***	**Results *V. splendidus* clade**
13/04/2016	**extra (Adult)**	23% (Up to 50%)	0+/30	22+/30	1+/30
26/04/2016	**IRTA-2016-001 (Juvenile)**	76% (Up to 90 %)	17+/30 (low infection intensity)	0+/30	28+/30
26/04/2016	**IRTA-2016-008 (Juvenile)**	46,6% (Up to 80%)	21+/30 (high infection intensity)	4+/30	27+/30
19/07/2016	**IRTA-2016-015 (Juvenile)**	3% (ligth acomulative mortality)	1+/30 (low infection intensity)	0+/30	4+/30
19/07/2016	**IRTA-2016-022 (Juvenile)**	no current mortality	0+/30	0+/30	1+/30
24/11/2016	**IRTA-2016-029 (Juvenile)**	30% recent mortality	7+/30 (moderate infection intensity)	1+/30	29+/30
25/01/2017	**IRTA-2017-001 (Juvenile)**	no mortality	0+/30	0+/30	30+/30
26/01/2017	**IRTA-2017-008 (Juvenile)**	no mortality	0+/30	0+/30	30+/30
20/04/2017	**IRTA-2017-015**	73.4% mortality	14+/30	*pending*	*pending*
25/04/2017	**IRTA-2017-022**	51.9% mortality	15+/30	*pending*	*pending*
05/05/2017	**extra (Juvenile)**	87.83% mortality	10+/18	0+/18	15+/18
05/05/2017	**extra (Juvenile)**	no mortality	0+/30	0+/30	19+/30
31/05/2017	**extra (Adult)**	85% mortality	0+/30	9+/30	24+/30

From Dungarvan bay, during outbreaks from 2016 to 2017, were collected also samples of other bivalves (27 *Littorina littorea*, 5 clams and 1 cockle) and environment matrix (14 from sediment and 3 of marine water). The screening of these samples as show the almost ubiquitary presence of Vibrios (except 9 *Littorina littorea* and 1 clam) while were totally absent *V. aestuarianus* and *V. coralliilyticus* (Table 10).

Date	VIVALDI Code	Sample	Vibrio spp	Results *V. aestuarianus*	Results *V. splendidus* clade	Results *V. coralliilyticus*
05/07/2016	MI-2016-001	Sediment	4+/4	0+/4	4+/4	0+/4
		Filtered water (plankton)	1+/1	0+/1	1+/1	0+/1
		Littorina littorea	13+/20	0+/20	15+/20	0+/20
03/10/2016	MI-2016-002	Sediment	6+/6	0+/6		
		Filtered water (plankton)	1+/1	0+/1		
10/01/2017	MI-2017-001	Sediment	4+/4	0+/4		
		Filtered water (plankton)	1+/1	0+/1		
		Littorina littorea	5+/7	0+/7		
		Cockles	1+/1	0+/1		
		Clams	4+/5	0+/5		

Table 10. Summary of screening results of samples collected from C. gigas aquaculture in Dungarvan bay during oyster mortality outbreaks.

Microbiota analysis

For microbiota analysis, 12 contrasting *C. gigas* samples were selected in Dungarvan bay (10 samples) and Ebro delta (2 samples) based on results from PCR analysis. In particular, most interesting sampling periods were chosen (*e.g.* periods with high or absence of mortality) and for each period 10 contrasting oyster samples were selected (5 samples infected by OsHV-1 or *V. aestuarianus* and 5 non-infected control samples).

From the sequences produced by Ion Torrent sequencing were removed barcode and adapters sequences, and raw sequences were trimmed to minimize bias associated with PCR amplification of target genes. Random sequencing errors were adjusted by removing reads that contained one or more ambiguous bases, had errors in the barcode or primer sequence, were atypically short (100 bp), and had an average quality score <0.05 according to CLC default settings.

To assess members of the microbiota community, phylogenetic identity of generated sequences from bivalve samples was analyzed using the CLC Microbial Genomics Module (v.11.0.1). Trimmed reads were first clustered at 97% similarity resulting in operational taxonomical units (OTUs). Singletons (OTUs with a single read in the data set) were excluded from the analysis. OTUs sequences were BLASTed against SILVA reference database (SILVA 16S, v123, 97%). The results of BLASTN were used to estimate the taxonomic content of the data set, using SILVA taxonomy with the CLC software. Only reads occurring at least five times in the trimmed data set were assigned to bacterial taxa and included in the results.

In general, the composition of the bacterial community associated to *C. gigas* was dominated by the classes of Gamma and Alphaproteobacteria followed by Epsilonproteobacteria, Mollicutes and Flavobacteria (data not shown).

As showed in Figure 17, the infection of one pathogen (*i.e.* OsHV-1 or *V. aestuarianus*) reduce the microbial diversity compared to controls. In details, the OsHV-1 infected oysters showed an increased presence of the genus *Vibrio* while the *C. gigas* infected by *V. aestuarianus* had in higher abundance of *Vibrio* and *Arcobacter*.

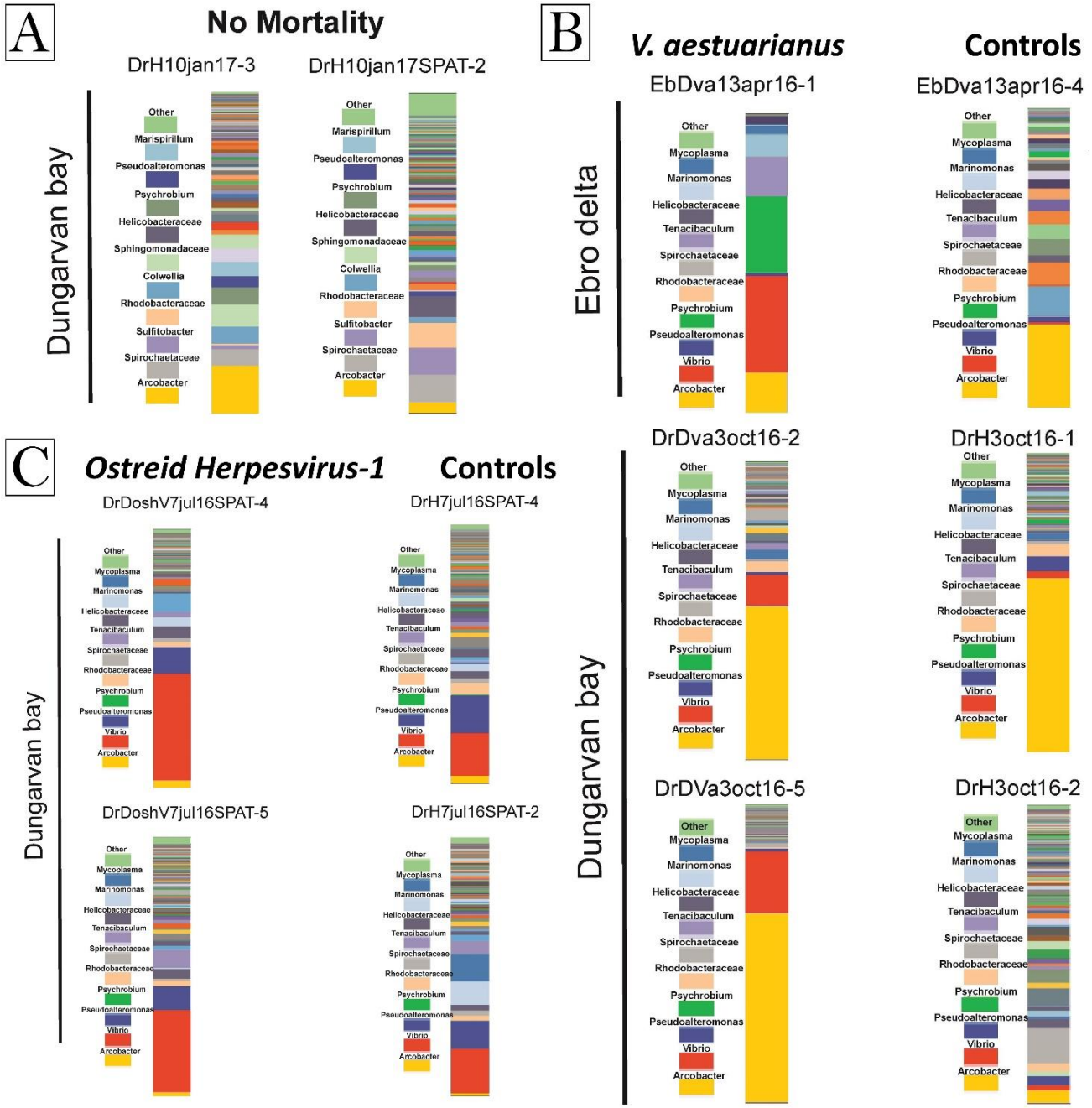

Figure 17. Relative abundance of bacterial genera of 12 contrasting samples by 16rDNA gene-based profiling analysis. (A) Samples collected without mortalities. (B) and (C) Samples collected during mortality events respectively linked with V. aestuarianus and OsHV-1.

Pathobiota analysis

To run the analysis, a pathobiota sequence database containing 884 was built and used to produce 12,114 different biotinylated RNA baits for selective capturing. The sequences selected for the target enrichment of pathobiota included:

- Phylogenetic markers for search and identify *Vibrio* species and strain when it is possible
- Markers for virulence factors that allow to search and identify the main virulence factors of Vibrios pathogens for human or marine invertebrate (mainly corals and bivalves)
- Phylogenetic and virulence markers of other pathogen microorganisms (bacteria, virus and eukaryotes) connected with marine invertebrate disease (mainly for corals and bivalves)

Target	Taxonomy	Main Host	Marker gene	N° allelic variants	lenght (nt)
VIBRIO	*Vibrio* spp	Human, Animal	*gyrB*	243	400
(phylogenetic markers)	*Vibrio* spp.	Human, Animal	*recA*	204	400
	Vibrio spp.	Human, Animal	*atpA*	133	400
	Vibrio spp.	Human, Animal	*dnaJ*	56	400
	Vibrio spp.	Human, Animal	*pyrH*	113	400
	V. tasmaniensis	*Crassostrea gigas*	LGP32 probes	10	400
VIBRIO	*V. cholerae* O1 El Tor	Human	*ctxA*	1	777
(virulence markers)	*V. cholerae* O1 El Tor	Human	*ctxB*	1	375
	V. cholerae O139	Human	*ctxA-B*	1	938
	V. cholerae O1 el Tor	Human	*tcpA*	1	675
	V. cholerae O1 classical	Human	*tcpA*	1	675
	V. cholerae O1 el Tor	Human	*rstR*	1	339
	V. cholerae O1 classical	Human	*rstR*	1	336
	V. cholerae O139	Human	*wbf*	1	449
	V. cholerae O1 el Tor	Human	*gbpA*	1	400
	V. parahaemolyticus	Human	*toxR*	1	552
	V. parahaemolyticus	Human	*tdh, trh*	3	570
	V. vulnificus	Human	*vvhA*	1	1416
	V. vulnificus	Human	*rtxA1*	1	400
	V. tasmaniensis	*Crassostrea gigas*	*vsm* (LGP32 strain)	1	1824
	V. tasmaniensis	*Crassostrea gigas*	*ompU* (LGP32 strain)	1	400
	V. aestuarianus	*Crassostrea gigas*	*vam*	1	1836
	V. tapetis	*Ruditapes philippinarum*	*djlA*	1	1826
	V. coralliilyticus	*Paramuricea clavata*	*vcpA*	15	1824
	V. harveyi	Stony corals	*vhhA*	1	1260
	V. crassostreae	*Crassostrea gigas*	*R-5.7*	1	2397
	V. tubiashii	*Crassostrea gigas*	metalloprotease	1	1821
	Vibrio spp.	Human, Animal	*MSHA*	12	400
ARCOBACTER	*Arcobacter* spp.	Human	*gyrB*	27	400
NOCARDIA	*Nocardia crassostrea*	*Crassostrea gigas, Ostrea edulis*	*rpoB, hsp65, gyrB*	3	400
MARTELIA	*Martelia refringens*	*Ostrea edulis, Mytilus edulis, M. galloprovincialis*	ITS1O, ITS1M, probe	3	400
BONAMIA	*Bonamia ostrea*	*Ostrea edulis*	5.8S-ITS rDNA, *hsp90* , *act1*	3	400
OsHV-1	Ostreid herpesvirus 1	*Crassostrea gigas*	C2/C6 (2), IA1-IA2, orf4, Hyp. Protein, RING fingerprotein gene	7	400
	Ostreid herpesvirus 1	*Crassostrea gigas*	ORF100	1	198
	Ostreid herpesvirus 1	*Crassostrea gigas*	C9-C10	1	197
	Ostreid herpesvirus 1	*Crassostrea gigas*	B3-B4	1	207
ENTEROCOCCUS	*E. faecalis, E. faecium, E. avium, E. gallinarum, E. casseliflavus, E. durans, E. raffinosus, E. mundtii*	Human	*atpA*	8	400
ROSEOVARIUS	*Roseovarius crassostrea*	*Crassostrea virginica*	*dnaJ, pyrH*	6	400
ESCHERICHIA	*Escherichia coli*	Human	*dnaJ, pyrH, atpA, gyrB*	4	400
ASPERGILLUS	*Aspergillus sydowii*	*Gorgonia ventalina* , Human	TUB2, *trpC* , ITS, calmodulin gene	4	400
AURANTIMONAS	*Aurantimonas coralicida*	Corals	*atpD, gyrB, recA, rpoB*	4	400
SERRATIA	*Serratia marcescens*	*Acropora palmata*	*gyrB, recA, dnaJ*	3	400
PSEUDOALTEROMONAS	*Pseudoalteromonas* sp.	*Rhopaloeides odorabile*	*gyrB*	1	400
			TOTAL	**884**	**29292**

Table 11. All the genes selected for target enrichment of the pathobiota.

A target enrichment next-generation sequencing protocol was for the first time applied for high taxonomic resolution analysis of the bivalve pathobiota on a total of 12 selected contrasting oyster samples including *C. gigas* samples collected during mortality episodes infected and non-infected by *V. aestuarianus* or OsHV1 as well as samples collected in the absence of mortality. A mock community sample (positive control) and a sterile seawater sample (negative control) were also included in the analysis (see methods section for details).

Following target enrichment and sequencing of DNA libraries a total of 67.614.544 sequence reads were produced with an average of 5.634.545 sequence reads produced for each sample. On average less than 5% of the reads mapped against reference sequences from the pathobiota database and were used to produce consensus sequences for pathobiota taxonomic classification with the NCBI blast function.

Results of the target enrichment protocol allowed for the first time the detection and relative quantification of members of the bivalve pathogen community in oyster tissues at the species-level taxonomic resolution. Generally, although the community of potentially pathogenic species includes different species in all samples a dominance of primary pathogens such as *V. aestuarianus* and OsHV-1 were observed in samples collected during mortality episodes that were linked to infections due to these pathogens (Figure 17).

In particular, *C. gigas* samples collected during mortality episodes of adult oysters linked to *V. aestuarianus* outbreaks in both Ebro delta and Dungarvan bay (*e.g.* samples EbDva13apr16-1, DrDva3oct16-2, DrDva3oct16-5) showed a large proportion of reads specifically mapping on *V. aestuarianus* phylogenetic and virulence marker sequences accounting on average for more than 40% of the total reads sequences. Accordingly, consensus sequences obtained by read mapping for the main marker genes (e.g. *gyrB*, *recA*, *atpA*, *dnaJ*, *pyrH*) including the *vam* gene encoding a zinc metalloprotease were unequivocally assigned to the species *V. aestuarianus* by the BLAST sequence analysis.

Interestingly, a significant proportion of reads common to all *V. aestuarianus* infected samples were also specifically mapping on *Arcobacter haliotis* marker sequences (*gyrB*). Although at lower relative abundance other species identified in these samples included *Vibrio alginolyticus*, *V. coralliilyticus, V. crassostrea, V. mediterranei, V. toranzoniae, V. splendidus* and *V. tasmaniensis.* Virulence genes such as metalloproteases (*V. splendidus vsm*, *V. coralliilyticus vcpA*) and *ompU* of *V. tasmaniensis* were also detected (Figure 17A).

In the OsHV-1 infected *C. gigas* samples collected during Dungarvan bay outbreaks (*e.g.* samples DrDoshV7jul16SPAT-4 and DrDoshV7jul16SPAT-5) around 70% of all the reads mapping on OsHV-1 genome. The infected samples showed also high relative abundance of *Splendidus* clade reads, specifically the virulence factor *vsm* in one sample. Although at lower relative abundance other species identified in these samples, with relative abundance differences them, included: *V. coralliilyticus, V. tasmaniensis, V. cyclotrophicus, V. alginolyticus, V. lentus, V. crassostrea, V. cortegadensis, V. pomeroyi*. Virulence genes such *V. tasmaniensis* and *V. splendidus vsm* and *ompU* of *V. tasmaniensis* were also detected (Figure 17B).

Interesting the pathobiota was successfully enriched also in one non-infected sample collected during OsHV-1 outbreaks, DrH7jul16SPAT-4. The two species *V. tasmaniensis* and *V. splendidus*

were found with high relative abundance, around 40%; in detail, were found also the virulence factors *vsm* and *ompU* (Figure 17B).

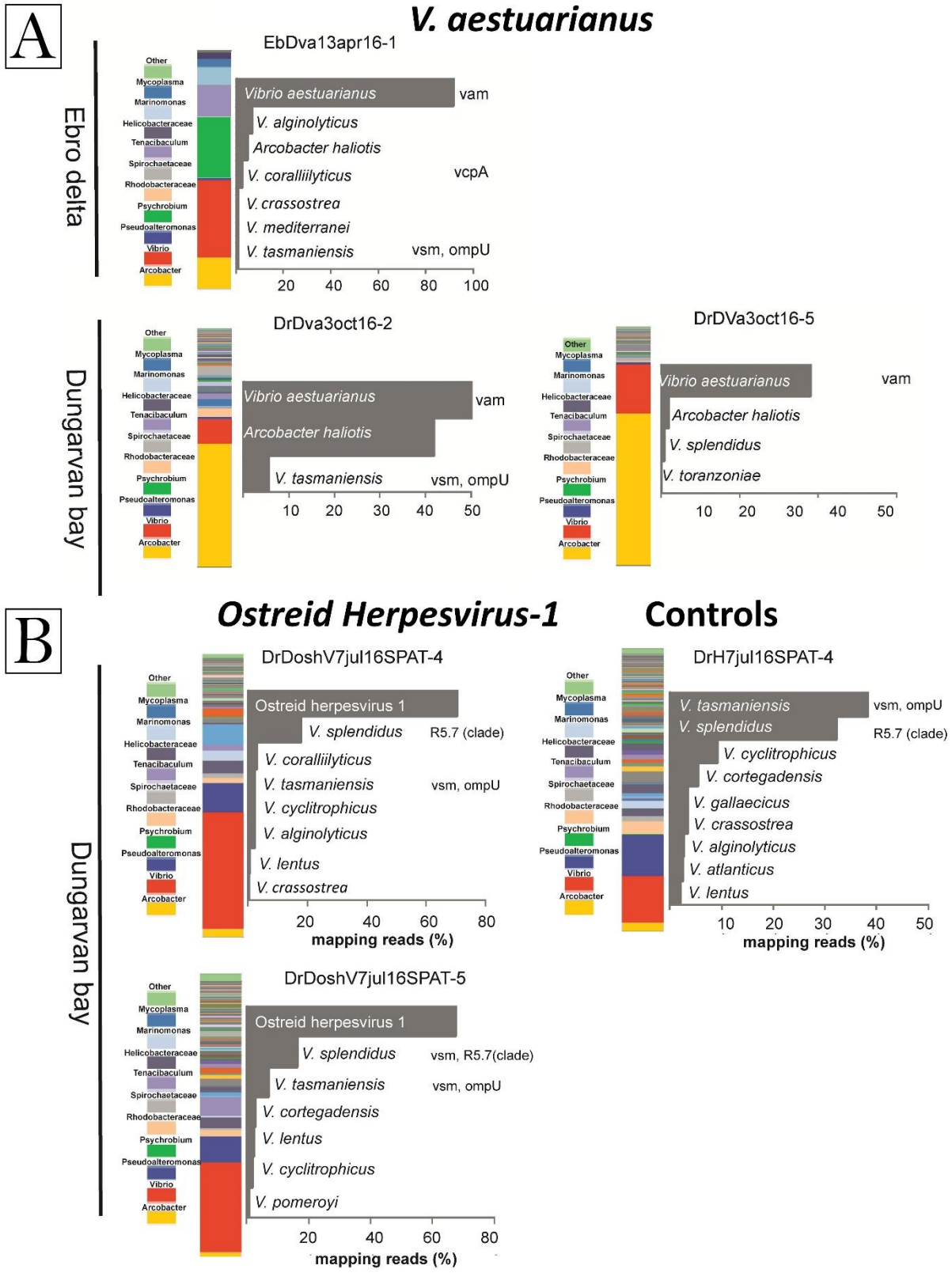

Figure 17. Pathobiota of the C. gigas samples during outbreaks linked with the 2 pathogens expressend in % mapping reads. 6 samples are not shown because they did not reveal pathobiota. (A) The samples of infected oysters during an event linked at V. aestuarianus. (B) Samples of 2 infected oysters and 1 control collected during an outbreaks caused by OsHV-1.

The synthetic community used as method control was successfully enriched: all the pathogens used for the sample preparation were found (Figure 18).

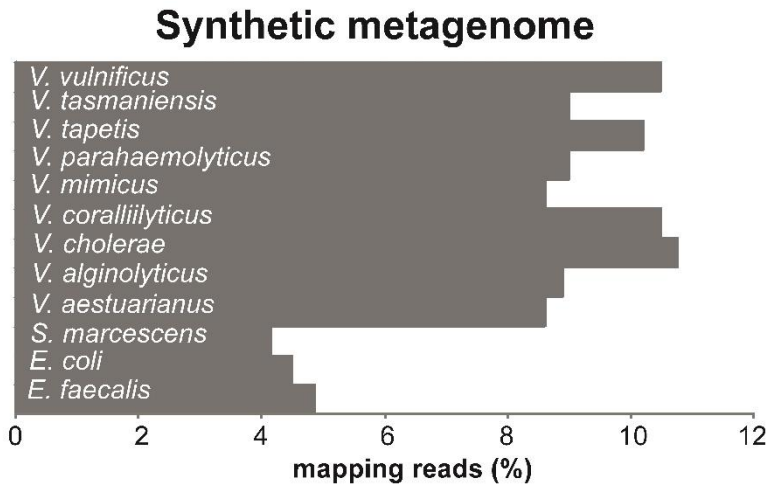

Figure 18. Target enrichment result of the synthetic community.

Discussions

This part of the work investigated for the first time the microbiota and pathobiota associated to farmed *C. gigas* at a European scale and provided new data on its composition and pattern of variability with particular reference to the occurrence of abnormal mortality episodes. In general, the composition of the bacterial community of *C. gigas* was dominated by the classes of Gamma and Alphaproteobacteria followed by Epsilonproteobacteria, Mollicutes and Flavobacteria.
Analysis of contrasting bivalve samples based on the presence/absence of microbial pathogens showed that an increase in abundance of bacteria belonging to the genus *Vibrio* and *Arcobacter* was observed in *V. aestuarianus* infected oysters compared to non-infected animals. An increase in the *Vibrio* fraction was also observed in juvenile oysters infected by OsHV-1 compared to non-infected oysters indicating that this bacterial group might play an important role in disease development. A significant change in the microbiota biodiversity was observed in adult oysters infected by *V. aestuarianus* where alpha diversity was significatively lower than those measured in either OsHV-1 infected or not infected oysters.

It is thus apparent that specific microbial taxa, and especially member of the *Vibrio* genus are likely to play a role in affecting oyster health status during disease outbreaks. Nevertheless, whether the condition of dysbiosis is a prerequisite for oyster infection or it is a consequence of developing disease it is difficult to discern. A recent study carried out by Lemire *et al.,* (2015) using high throughput experimental infection assays coupled with microbial cultivation, comparative genomics and molecular genetics showed that the onset of disease in oysters is associated with progressive replacement of diverse benign bacterial colonizers by members of a phylogenetically coherent virulent population. According to their results dysbiosis might be seen as a new form of polymicrobial disease, in which a population/consortium of virulent strains but also nonpathogenic strains contribute to oyster mortality (Lemire *et al.,* 2015). Interestingly bacteria

were observed to be necessary for the disease during OsHV-1 infection outbreak while the herpes virus appears neither essential nor sufficient to cause oyster deaths.

A larger number of microbial pathogens (hereinafter referred to as the “pathobiota”) than previously thought might thus be involved in the establishment of oyster infection and development of diseases. Unfortunately, investigating the C gigas pathobiota at high level of taxonomic resolution is hardly achievable by culturable methods or classic molecular methods. In fact several environmental strains are impossible to cultivate in the laboratory due to demanding grown requirements, moreover up to date molecular approaches such as PCR analysis, 16S rDNA profiling and shotgun metagenomic sequencing have limits for the detection and genotyping of selected microorganisms (*e.g.,* potential pathogens) within the microbiota (*e.g.*, due to inhibitor presence, low concentrations of some genomes, hampering by host DNA). To avoid these limits, we developed a process for target enrich and NGS the microbiome component of interest, the pathobiota.
This method was developed following the approach previously applied for the target sequencing of *V. cholerae* DNA in complex environmental samples (Vezzulli *et al.*, 2017). A new target enrichment NGS based protocol for the deep taxonomic profiling of the pathogenic community associated to bivalves was developed and for the first time was applied on selected *C. gigas* samples. The protocol is based on the use of biotinylated RNA baits (on average >100-mer) for selective capturing of nearly one thousand phylogenetic and virulence markers targeting the Vibrio community and other potential pathogenic microorganisms in oyster tissues via hybridization.
By this new method it was possible to detect and relatively quantify potential members of the oyster associated microbial pathogenic community in infected oysters at the species-level taxonomic resolution. The pathobiota of the infected *C. gigas* showed complex communities with several microbial species involved. In particular, the *V. aestuarianus* infected oysters showed always the presence also of *A. haliotis* and other *Vibrio* spp. of *Splendidus* clade and some *vibrio* virulence factors. The OSHV-1 infection demonstrated an important abundance of Vibrios in the pathobiota, with high presence of *V. splendidus* while *A. haliotis* was absent in all the samples.

In conclusions, we observed the reduction of the microbiome biodiversity in infected *C. gigas* and we successfully isolate and identified at the species-level taxonomic resolution the pathogenic community, “pathobiota”, by using an innovative and efficient method of target enrichment and NGS. The success of this analysis showed the potentiality of this new approach for study the complexity of the outbreak events in Pacific oyster farming areas.

FINAL CONCLUSIONS

The *C. gigas* mortality events in European farming areas are linked with several factors including microbiological agents; although some microbial pathogens have been identified (*e.g., V. aestuarianus* and OsHV-1) the mechanisms and processes driving oyster infections and progression of diseases are still poorly understood. Bacteria belonging to the genus *Vibrio* are known to play a role in such processes.

Considering the ability of *Vibrio* to accumulate on planktonic elements in the marine environment (*i.e.,* chitin as free fragments, marine snow, exoskeleton of arthropods), this study showed that some plankton matrices mediating *V. aestuarianus* infections in the pacific oyster *C. gigas*. In particular results from artificial infection experiments, have shown that phytoplankton cells (*N. gaditana*) and marine snow particles significantly promote *V. aestuarianus* 02/041 intake by *C. gigas* maintained under stressful conditions in the laboratory. Such intake is associated with a decrease in Lysosomal membrane stability of oyster hemocyte indicating a compromised health status of infected oysters. In contrast, chitin particles did not appear to favor pathogen transmission to the bivalve host. Phytoplankton cells and marine snow particles both constitute a food source for *C. gigas* and can be filtered efficiently by the bivalve filtration system (*e.g.,* due to optimal size and chemical properties). Pathogenic bacteria such as *V. aestuarianus* 02/041 might take advantage of this by adhering to food particles and entering the bivalve host more efficiently. Bacteria attached to environmental substrates could also be less sensitive to bivalve antimicrobial activity (as reported for *Vibrio* species involved in human infections), although at present this interpretation remains speculative.

A new target enrichment next generation sequencing protocol was also developed and successfully applied to explore the Pacific Oyster pathobiota during mortality episodes in Europe. The protocol allowed for the first time the identification and relative quantification of potential pathogenic microorganisms most of which belonging to the genus *Vibrio* which are could be potentially involved in polymicrobial infections affecting *C. gigas* during mass mortality episodes.

In conclusion, besides significance in the field of basic research the obtained results might also have more practical implications: *e.g.* for the monitoring and evaluation of the infection risk in oyster farming areas by improving pathogens detection and the prediction of their transmission routes

ABBREVIATIONS

ASW	Artificial Seawater Composition: NaF 1.9 mg/l; SrCl2*6H2O 13 mg/l; H3BO3 20 mg/l; KBr 67 mg/l; KCl 466 g/l; CaCl2*2H2O 733 mg/l; Na2SO4 2.66 g/l; MgCl2*6H2O 3.33 g/l; NaHCO3 133 mg/l; NaCl 27.65 g/l; pH 8
CFU	Colony Forming Unit
GBPA	N-acetyl Glucosamine-Binding Protein A
LB	Luria Bertani
LMS	Lysosomal Membrane Stability
MSHA	Mannose-Sensitive HemAgglutinin
NGS	Next Generation Sequencing
NRRT	Neutral Red Retention Time
OsHV-1	Ostreid Herpesvirus 1
PBS	Phosphate Buffered Saline Composition: NaCl 8 g/l; KCl 0.2 g/l; Na2HPO4.12H2O 3.62 g/l; KHPO4 0.24 g/l
Ppt	Parts Per Thousands
RT-PCR	Real -Time Polymerase Chain Reaction
TCBS	Thiosulfate Citrate Bile salts sucrose
vam	*Vibrio aestuarianus* metalloprotease
vsm	*Vibrio splendidus* metalloprotease

BIBLIOGRAPHY

Alfaro A. C., Nguyen T. V. and Merien F. (2018) The complex interactions of *Ostreid herpesvirus 1*, *Vibrio* bacteria, environment and host factors in mass mortality outbreaks of *Crassostrea gigas*. Rev Aquacult. doi:10.1111/raq.12284

Auby I., Maurer D. (2004) Etude de la reproduction de l'huîtrecreusedans le Bassin d'Arcachon http://archimer.ifremer.fr/doc/00040/15145/

Austin B., Austin D., Sutherland R., Thompson F., Swings J. (2005) Pathogenicity of vibrios to rainbow trout (Oncorhynchus mykiss, Walbaum) and *Artemia nauplii*. Environ Microbiol. 7(9):1488-95.

Azandegbe A., Garnier M., Andrieux-Loyer F., Kerouel R., Philippon X. and Nicolas J.L. (2010) Occurrence and seasonality of *Vibrio aestuarianus* in sediment and *Crassostrea gigas* haemolymph at two oyster farms in France. Diseases of Aquatic Organisms,91, 213-221.

Azéma P., Travers M.A., Benabdelmouna A., Dégremont L. (2016) Single or dual experimental infections with *Vibrio aestuarianus* and OsHV-1 in diploid and triploid *Crassostrea gigas* at the spat, juvenile and adult stages. Journal of Invertebrate Pathology, Volume 139, 2016, Pages 92-101, ISSN 0022-2011.

Balbi T., Fabbri R., Montagna M., Camisassi G., Canesi L. (2017) Seasonal variability of different biomarkers in mussels (Mytilus galloprovincialis) farmed at different sites of the Gulf of La Spezia, Ligurian sea, Italy. Mar Pollut Bull. 116(1-2):348-356

Baumann P., Furniss A.L., Lee J.V. Genus 1. Vibrio Pacini 1854, 411AL Krieg N.r., Holt J.g. (Eds.), Bergey's .'Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore (1984), pp. 518-538

Boles B.R., Horswill A.R. (2012) Swimming cells promote a dynamic environment within biofilms. *Proc Natl Acad Sci U S A*. 2012;109(32):12848-9.

Castaños C., Pascual M., Camacho A.P. (2009) Reproductive biology of the nonnative oyster, *Crassostrea gigas* (Thunberg, 1793), as a key factor for its successful spread along the rocky shores of Northern Patagonia, Argentina. J. Shellfish Res., 28 (2009), pp. 837-847

Chávez-Villalba J., Mingant C., Cochard J.C., Le Pennec M. (2001) Gamétogenèse chez l'huître Crassostrea gigas de l'AberBenoît (Bretagne, France), à la limitenord de son aire de reproduction. Haliotis, 30 (2001), pp. 1-12

Chakraborty S., Nair G.B., and Shinoda S. (1997) Patho-genic vibrios in the natural aquatic environment. *Rev Environ Health* 12: 63–80.

Chiavelli D. A., Marsh J. W. & Taylor R. K. (2001) The mannose-sensitive hemagglutinin of *Vibrio cholerae* promotes adherence to zooplankton. *Applied and environmental microbiology*, *67*(7), 3220-5.

Clerissi C., Lorgeril J., Petton B., Lucasson A., Escoubas J.M., Gueguen Y., Mitta G., Toulza E. (2018) Diversity and stability of microbiota are key factors associated to healthy and diseased *Crassostrea gigas* oysters. bioRxiv 378125; doi: https://doi.org/10.1101/378125

Cockburn T. A. & Cassanos J. G. (1960) Epidemiology of endemic cholera. *Public Health Reports*, *75*(9), 791–804.

Cognie B., Haure J., Barillé L. (2006) Spatial distribution in a temperate coastal ecosystem of the wild stock of the farmed oyster *Crassostrea gigas* (Thunberg). Aquaculture, 259 (2006), pp. 249-259

Colwell R. R. and HUQ A. (1994) Environmental Reservoir of *Vibrio cholerae* The Causative Agent of Cholera. Annals of the New York Academy of Sciences, 740: 44-54 doi:10.1111/j.1749-6632.1994.tb19852.x-

Colwell, R.R. (2002) A voyage of discovery: cholera, climate and complexity. Environ Microbiol 4*:* 67–69*.*

Davey M.E., O'toole G.A. (2000) Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev*. 2000;64(4):847-67.

De Decker S., Saulnier D. (2011) Vibriosis induced by experimental cohabitation in *Crassostrea gigas*: evidence of early infection and down-expression of immune-related genes. Fish Shellfish Immunol 30:691–699

De Decker S., Normand J., Saulnier D., Pernet F., Castagnet S. and Boudry P. (2011) Responses of diploid and triploid Pacific oysters *Crassostrea gigas* to Vibrio infection in relation to their reproductive status. Journal of Invertebrate Pathology,106, 179-191

De Decker S., Reynaud Y., Saulnier D. (2013) First molecular evidence of cross-species induction of metalloprotease gene expression in *Vibrio* strains pathogenic for Pacific oyster *Crassostrea gigas* involving a quorum sensing system. Aquaculture, Volumes 392–395,2013,Pages 1-7,ISSN 0044-486,

De Kantzow M., Hick P., Becker J.A., Whittington R.J. (2016) Effect of water temperature on mortality of Pacific oysters *Crassostrea gigas* associated with microvariant ostreid herpesvirus 1 (OsHV-1 µVar). Aquacult Environ Interact 8:419-428. https://doi.org/10.3354/aei00186

Dégremont L., Azéma P. and Travers M.A. (2014a) Spat and adult mortality related to *Vibrio aestuarianus* in *Crassostrea gigas* in France. Proceedings of the National Shellfish Association Meeting, Jacksonville, Florida, USA, March 29–April 2, 2014.

Dégremont L., Lamy J. B., Pépin J. F., Travers M. A. & Renault T. (2015) New Insight for the Genetic Evaluation of Resistance to *Ostreid Herpesvirus* Infection, a Worldwide Disease, in *Crassostrea gigas*. *PloS one*, *10*(6), e0127917. doi:10.1371/journal.pone.0127917

Domeneghetti S., Varotto L., Civettini M., Rosani U., Stauder M., Pretto T., Pezzati E., Arcangeli G., Turolla E., Pallavicini A., Venier P. (2014) Mortality occurrence and pathogen detection in *Crassostrea gigas* and *Mytilus galloprovincialis* close-growing in shallow waters (Goro lagoon, Italy). Fish & Shellfish Immunology, Volume 41, ssue 1,2014,Pages 37-44,ISSN 1050-4648,

Donlan R.M. & Costerton J.W. (2002) Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. *Clinical Microbiology Reviews*. 2002;15(2):167-193. doi:10.1128/CMR.15.2.167-193.2002.

Duperthuy M., Schmitt P., Garzón E., Caro A., Rosa R. D., Le Roux F., Lautrédou-Audouy N., Got P., Romestand B., de Lorgeril J., Kieffer-Jaquinod S., Bachère E., Destoumieux-Garzón D. (2011). Use of OmpU porins for attachment and invasion of Crassostrea gigas immune cells by the oyster pathogen *Vibrio splendidus*. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(7), 2993-8.

Dutertre M., Beninger P.G., Barillé L., Papin M., Rosa P., Barillé A.-L., Haure J. (2009) Temperature and seston quantity and quality effects on field reproduction of farmed oysters, *Crassostrea gigas*, in Bourgneuf Bay, France. Aquat. Living Resour., 22 (2009), pp. 319-329

EFSA Panel on Animal Health and Welfare (AHAW). «Oyster Mortality: Oyster Mortality». *EFSA Journal* 13, n. 6 (june 2015): 4122. https://doi.org/10.2903/j.efsa.2015.4122.

Eiler A., Johansson M. and Bertilsson S. (2006) Environmental influences on Vibrio populations in northern temperate and boreal coastal waters (Baltic and Skagerrak Seas). Applied and Environmental Microbiology, 72, 6004-6011.

Enríquez-Díaz M., Pouvreau S., Chávez-Villalba J., Pennec M. (2009) Le Gametogenesis, reproductive investment, and spawning behavior of the Pacific giant oyster *Crassostrea gigas*: evidence of an environment-dependent strategy**.** Aquac. Int., 17 (2009), pp. 491-506

Estes R.M., Friedman C.S., Elston R.A., Herwig R.P. (2004) Pathogenicity testing of shellfish hatchery bacterial isolates on Pacific oyster *Crassostrea gigas* larvae. Dis Aquat Organ. 2004 Mar 10;58(2-3):223-30.

Fabioux C., Huvet A., Souchu P., Le Pennec M., Le Pouvreau S. (2005) Temperature and photoperiod drive *Crassostrea gigas* reproductive internal clock. Aquaculture, 250 (2005), pp. 458-470

FAOSTAT, 2012. Food and Agriculture Organization of the United Nations, Statistical Yearbook, Rome.

FAO 2014, Cultured Aquatic Species Information Programme

Farmer J.J. & Hickman-Brenner F.W. (2006) The Genera *Vibrio* and *Photobacterium*. In: Dworkin M., Falkow S., Rosenberg E., Schleifer KH., Stackebrandt E. (eds) The Prokaryotes. Springer, New York, NY

Froelich B., Ayrapetyan M., Oliver J. D. (2013) Integration of *Vibrio vulnificus* into marine aggregates and its subsequent uptake by *Crassostrea virginica* oysters. Enviorn Microbiol 49(13), 1454–1458

Friedman C.S., Estes R.M., Stokes N.A., Burge C.A., Hargove J.S., Barber B.J., Elston R.A., Burreson E.M., Reece K.S. (2005) Herpes virus in juvenile Pacific oysters *Crassostrea gigas* from Tomales Bay, California, coincides with summer mortality episodes. Dis Aquat Organ.2005 Jan 25;63(1):33-41.

Gauthier M.J. (2000) Nonculturable microorganisms in the environment (pp 87-112) Washington D.C.: American Society for Microbiology

Garnier M., Labreuche Y., Garcia C., Robert A. and Nicolas J.L. (2007) Evidence for the involvement of pathogenic bacteria in summer mortalities of the Pacific oyster *Crassostrea gigas*. Microbial Ecology,53, 187-196.

Garnier M., Labreuche Y., e Nicolas J.L. (2008) Molecular and Phenotypic Characterization of *Vibrio aestuarianus* Subsp. Francensis Subsp. Nov. a Pathogen of the Oyster *Crassostrea gigas*. *Systematic and Applied Microbiology* 31, n. 5 (2008): 358–65.

Gerdes D. (1983) The Pacific oyster *Crassostrea gigas*: Part I. Feeding behaviour of larvae and adults. Aquaculture, Volume 31, Issues 2–4, 1983, Pages 195-219, ISSN 0044-8486,

Go J., Deutscher A.T., Spiers Z.B., Dahle K., Kirkland P.D., Jenkins C. (2017) Mass Mortalities of Unknown Aetiology in Pacific Oysters *Crassostrea Gigas* in Port Stephens, New South Wales, Australia. *Diseases of Aquatic rganisms* 125, n. 3 (august 2017): 227–42.

Goudenège D., Travers M.A., Lemire A., Haffner P., Labreuche Y., Petton B., Mangenot S., Calteau A., Mazel D., Nicolas J.L., Jacq A. and Le Roux F. (2015) A single regulatory gene is sufficient to alter *Vibrio aestuarianus* pathogenicity in oysters. Environmental Microbiology.

Hsieh J.L., Fries J.S. and Noble R.T. (2008) Dynamics and predictive modelling of Vibrio spp. in the Neuse River Estuary, North Carolina, USA. Environmental Microbiology,10, 57-64.

Huq A., Small E.B., West P.A., Huq M.I., Rahman R., Colwell R.R. (1983) Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. *Appl Environ Microbiol*. 1983;45(1):275-83.

Huq A., Whitehouse C.A., Grim C.J., Alam M., Colwell R.R. (2008) Biofilms in water, its role and impact in human disease transmission. Current Opinion in Biotechnology, Volume 19, Issue 3, 2008, Pages 244-247, ISSN 0958-1669.

Igbinosa O. E., Okoh A. I. (2008) Emerging Vibrio species: an unending threat to public health in developing countries. Research in Microbiology, Volume 159, Issues 7–8,2008,Pages 495-506, ISSN 0923-2508.

Judah L. (2002) Phytoplankton community structure and seasonal succession in Tomales Bay, California. M. Sc Thesis, San Francisco State University, San Francisco, California, USA, California.

Kamiyama T. (2011) Microzooplankton as a food source for the Pacific oyster *Crassostrea gigas*: seasonal variation in gut contents and food availability. Fish Sci (2011) 77:961–974

Keeling S.E., Brosnahan C.L., Williams R., Gias E., Hannah M., Bueno R., McDonald W.L. and Johnston C. (2014) New Zealand juvenile oyster mortality associated with ostreid herpesvirus 1-an opportunistic longitudinal study. Diseases of Aquatic Organisms,109, 231-239.

Kierek K., & Watnick P. I. (2003). The *Vibrio cholerae* O139 O-antigen polysaccharide is essential for Ca2+-dependent biofilm development in seawater. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(24), 14357-62.

Kirn T.J., Jude A.B. & Taylor R.K. (2005) A colonization factor links *Vibrio cholerae* environmental survival and human infection *Nature* volume 438, pages 863–866 (08 December 2005)

Knowles G., Handlinger J., Jones B., Moltschaniwskyj N. (2014) Hemolymph chemistry and histopathological changes in Pacific oysters (*Crassostrea gigas*) in response to low salinity stress. Journal of Invertebrate Pathology, 121, pp. 78-84. DOI: 10.1016/j.jip.2014.06.013

Labreuche Y., Le Roux F., Henry J., Zatylny C., Huvet A., Lambert C., Soudant P., Mazel D. and Nicolas J.L. (2010) *Vibrio aestuarianus* zinc metalloprotease causes lethality in the Pacific oyster *Crassostrea gigas* and impairs the host cellular immune defenses. Fish & Shellfish Immunology, 29, 753-758.

Le Deuff R.M., Renault T. Purification and partial genome characterization of a herpes-like virus infecting the Japanese oyster, Crassostrea gigas. J Gen Virol. 1999 May; 80 (Pt 5):1317-22.

Le Roux F., Labreuche Y., Davis B.M., Iqbal N., Mangenot S., Goarant C., Mazel D. and Waldor M.K. (2011) Virulence of an emerging pathogenic lineage of *Vibrio nigripulchritudo* is dependent on two plasmids. Environmental Microbiology,13, 296-306.

Le Roux F., Binesse J., Saulnier D., & Mazel D. (2006). Construction of a Vibrio splendidus mutant lacking the metalloprotease gene vsm by use of a novel counter selectable suicide vector. *Applied and environmental microbiology*, *73*(3), 777-84.

Lemire A., Goudenège D., Versigny T., Petton B., Calteau A., Labreuche Y., Le Roux F. (2015) Populations, not clones, are the unit of vibrio pathogenesis in naturally infected oysters (2015) ISME Journal, 9 (7), pp. 1523-1531.
Li K., Bihan M., Methé B.A. (2013) Analyses of the stability and core taxonomic memberships of the human microbiome. *PLoS One*. 2013;8(5):e63139. Published 2013 May 6.

Lipp E.K., Huq A., Colwell R.R. (2002) Effects of global climate on infectious disease: the cholera model. doi: 10.1128/CMR.15.4.757-770.2002 *Clin. Microbiol. Rev. October 2002 vol. 15 no. 4 757-770* 1 October 2002

Lokmer A., Wegner K.M. (2015) Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. ISME J 9:670–682

Mann R. (1979) Some biochemical and physiological aspects of growth and gametogenesis in *Crassostrea gigas* and *Ostrea edulis* grown at sustained elevated temperatures. J. Mar. Biol. Assoc. U. K., 59 (1979), pp. 95-110

Martínez-Gómez C., Bignell J., Lowe D. (2015) Lysosomal membrane stability in mussels (2015) ICES Tech. Mar. Environ. Sci, 56, p. 41.

Martínez-Gómez C., Fernández B., Robinson C., Campillo J., León V., Benedicto J., Hylland K., Vethaak A. (2016) Assessing environmental quality status by integrating chemical and biological effect data: The Cartagena coastal zone as a case. Marine Environmental Research. 124. 10.1016/j.marenvres.2016.04.008.

Meibom K.L., Li X.B., Nielsen A.T., Wu C.Y., Roseman S. & Schoolnik G.K. (2004) The *Vibrio cholerae* chitin utilization program. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(8), 2524-9.

Meibom K.L., Blokesch M., Dolganov N.A., Wu C.Y., Schoolnik G.K. (2005) Chitin Induces Natural Competence in *Vibrio cholerae. Science*16 Dec 2005 : 1824-1827

Montgomery M. T., & Kirchman, D. L. (1993). Role of Chitin-Binding Proteins in the Specific Attachment of the Marine Bacterium Vibrio harveyi to Chitin. *Applied and environmental microbiology*, *59*(2), 373-9.

Nalin D. R., Daya V., Reid A., Levine M. M., & Cisneros L. (1979) Adsorption and growth of Vibrio cholerae on chitin. *Infection and immunity*, *25*(2), 768-70.

Olafsen J.A., Mikkelsen H.V., Giæver H.M., Hansen G.H. (1993) Indigenous Bacteria in Hemolymph and Tissues of Marine Bivalves at Low Temperatures. Appl Environ Microbiol. 1993 Jun; 59(6): 1848–1854.

Oliver J. D., Nilsson L., & Kjelleberg S. (1991) Formation of nonculturable Vibrio vulnificus cells and its relationship to the starvation state. *Applied and Environmental Microbiology*, *57*(9), 2640–2644.

Osahor A., Deekonda K., Lee C.W., Sim E., Radu A., Narayanan K. (2017) Rapid preparation of adherent mammalian cells for basic scanning electron microscopy (SEM) analysis. Analytical Biochemistry, Volume 534,2017,Pages 46-48

OSPAR Commission, (2013). Background documents and technical annexes for biological effects monitoring. 239 pp. www.ospar.org.

Paillard C., Allam B., Oubella R. (2004) Effect of temperature on defense parameters in manila clam *Ruditapes philippinarum* challenged with *Vibrio tapetis*. Dis Aquat Organ. 11;59(3):249-62.

Payne S.M. & Finkelstein R.A. (1978). Siderophore production by *Vibrio cholerae*. *Infection and immunity*, *20*(1), 310-1.

Paul-Pont I., Dhand N.K., Whittington R.J. (2013) Spatial distribution of mortality in Pacific oysters *Crassostrea gigas*: reflection on mechanisms of OsHV-1 transmission. Dis Aquat Org 105:127-138.

Pernet F., Barret J., Le Gall P., Corporeau C., Dégremont L., Lagarde F., Pépin J., Keck N. (2012) Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practices in the Mediterranean Thau lagoon, France. Aquacult Environ Interact 2:215-237.

Petton B., Bruto M., James A., Labreuche Y., Alunno-Bruscia M., & Le Roux F. (2015) *Crassostrea gigas* mortality in France: the usual suspect, a herpes virus, may not be the killer in this polymicrobial opportunistic disease. *Frontiers in microbiology*, *6*, 686.

Pruzzo C. , Vezzulli L. and Colwell R. R. (2008) Global impact of *Vibrio cholerae* interactions with chitin. Environmental Microbiology, 10: 1400-1410. doi:10.1111/j.1462-2920.2007.01559.x

Pruzzo C., Tarsi R., Del Mar Lleò M., Signoretto C., Zampini M., Pane L., Colwell R. R. and Canepari P. (2003) Persistence of adhesive properties in *Vibrio cholerae* after long-term exposure to sea water. Environmental Microbiology, 5: 850-858. doi:10.1046/j.1462-2920.2003.00498.x

Pruzzo C., Crippa A., Bertone S., Pane L., Carli A. (1996) Attachment of *Vibrio alginolyticus* to chitin mediated by chitin-binding proteins Microbiology 142(8):2181-2186 doi:10.1099/13500872-142-8-2181

Rasmussen T., Rasmus Bugge J. and Ole S. (2007) The two chromosomes of *Vibrio cholerae* are initiated at different time points in the cell cycle. *The EMBO Journal* 26, n. 13 (11 luglio 2007): 3124.

Rees J., Pond K., Kay D., Bartram J., Domingo J.S. 2010; World Health Organization: Safe management of shellfish and harvest waters. IWA Publishing London, United Kingdom

Reguera G. & Kolter R. (2005) Virulence and the environment: a novel role for *Vibrio cholerae* toxin-coregulated pili in biofilm formation on chitin. *Journal of bacteriology*, *187*(10), 3551-5.

Ren J.S., Ross A.H., Schiel D.R. (2000) Functional descriptions of feeding and energetics of the Pacific oyster *Crassostrea gigas* in New Zealand. MEPS 208:119-130 (2000)

Romero A., Costa M.D., Forn-Cuni G., Balseiro P., Chamorro R., Dios S., Figueras A. and Novoa B. (2014) Occurrence, seasonality and infectivity of Vibrio strains in natural populations of mussels *Mytilus galloprovincialis*. Diseases of Aquatic Organisms,108, 149-163.

Rosenberg E., Koren O., Reshef L., Efrony R., Zilber-Rosenberg I. (2007) The role of microorganisms in coral health, disease and evolution. Nature Review Microbiology 5:355-362

Samain J.F. and McCombie H. (2008) Summer mortality of Pacific oyster *Crassostrea gigas*. The Morest Project. Versailles: Quae Editions. 379 pp. SAS Institute Inc. 1995. Basis statistics using SAS/STAT, software course notes. Cary, NC: SAS Institute. 720 pp.

Saulnier D., De Decker S., Haffner P., Cobret L., Robert M. and Garcia C. (2010) A Large-Scale Epidemiological Study to Identify Bacteria Pathogenic to Pacific Oyster *Crassostrea gigas* and Correlation Between Virulence and Metalloprotease-like Activity. Microbial Ecology, 59, 787-798.

Scarano C., Spanu C., Ziino G., Pedonese F., Dalmasso A., Spanu V., Virdis S. and De Santis E.P.L. (2014) Antibiotic resistance of Vibrio species isolated from *Sparus aurat* areared in Italian mariculture. New Microbiologica,37, 329-337.

Schikorski D., Renault T., Saulnier D., Faury N., Moreau P. and Pépin J.-F. (2011) Experimental infection of Pacific oyster *Crassostrea gigas* spat by *ostreid herpesvirus* 1: demonstration of oyster spat susceptibility. Veterinary Research2011 42:27 https://doi.org/10.1186/1297-9716-42-27

Saulnier D., De Decker S. and Haffner P. (2009) Real-time PCR assay for rapid detection and quantification of *Vibrio aestuarianus* in oyster and seawater: A useful tool for epidemiologic studies. Journal of Microbiological Methods, Volume 77, Issue 2, 2009, Pages 191-197

Singleton F. L., Attwell R., Jangi S., & Colwell R. R. (1982) Effects of temperature and salinity on *Vibrio cholerae* growth. *Applied and Environmental Microbiology*, *44*(5), 1047–1058.

Sumner J., De Paola A., Osaka K., Karunasager I., Walderhaug M., Bowers J. (2001) Hazard identification, exposure assessment and hazard characterization of Vibrio spp. in seafood. Joint FAO/WHO Activities on Risk Assessment of Microbiological Hazards in Foods. 1-108.

Tantillo G., Fontanarosa M., Di Pinto A. and Musti M. (2004) Updated perspectives on emerging vibrios associated with human infections. Letters in Applied Microbiology, 39: 117-126. doi:10.1111/j.1472-765X.2004.01568.x

Tarsi R., & Pruzzo C. (1999). Role of surface proteins in *Vibrio cholerae* attachment to chitin. *Applied and environmental microbiology*, *65*(3), 1348-51.

Tison D.L. and Seidler R.J. (1983) *Vibrio-Aestuarianus* – a New Species from Estuarine Waters and Shellfish. International Journal of Systematic Bacteriology, 33, 699-702.

Renault T. and Novo B. (2004) Viruses infecting bivalve molluscs. Aquat. Living Resour. 17 (2004) 397-409

Travers M.A., Boettcher Miller K., Roque A., Friedman C.S. (2015) Bacterial diseases in mirane bivalves. J Invertebr Pathol. 2015 Oct; 131:11-31. doi: 10.1016/j.jip.2015.07.010

Troost K., Kamermans P., Stamhuis E. & Wolff W.J. (2004) Are introduced oyster (*Crassostrea gigas*) hampering the recruitment of indigenous bivalve filter feeders? ICES conference 2004, United Kingdom, 01/07/2015, pp. 10.

Vethaak A.D., Davies I.M., Thain J.E., Gubbins M.J., Martínez-Gomez C., Robinson C.D., Moffat C.F., Burgeot T., Maes T., Wosniok W., Giltrap M., Lang T., Hylland K. (2015) Integrated indicator framework and methodology for monitoring and assessment of hazardous substances and their effects in the marine environment Mar. Environ. ResVolume 124, 2017, Pages 11-20, ISSN 0141-1136.

Vezzulli L., Pruzzo C., Huq A. and Colwell R.R. (2010) Environmental reservoirs of Vibrio cholerae and their role in cholera. Environmental Microbiology Reports (2010) 2(1), 27–33

Vezzulli L., Pezzati E., Stauder M., Stagnaro L., Venier P. and Pruzzo C. (2015) Aquatic ecology of the oyster pathogens *Vibrio splendidus* and *Vibrio aestuarianus*. Environmental Microbiology 17(4), 1065-1080.

Vezzulli L., Grande C., Reid P.C., Hélaouët P., Edwards M., Höfle M.G., Brettar I., Colwell R.R., Pruzzo C. (2016) Climate impact on Vibrio prokaryotes Proceedings of the National Academy of Sciences Aug 2016, 113 (34) E5062-E5071; DOI: 10.1073/pnas.1609157113

Vezzulli L., Stagnaro L., Grande C., Tassistro G., Canesi L., Pruzzo C. (2018) Comparative 16SrDNA Gene-Based Microbiota Profiles of the Pacific Oyster (*Crassostrea gigas*) and the Mediterranean

Mussel (*Mytilus galloprovincialis*) from a Shellfish Farm (Ligurian Sea, Italy) Microb Ecol (2018) 75: 495. https://doi.org/10.1007/s00248-017-1051-6

Vogeler S., Bean T.P., Lyons B.P., Galloway T.S. (2016) Dynamics of Nuclear Receptor Gene Expression during Pacific Oyster Development. *BMC Developmental Biology* 16, n. 1 (dicembre 2016). https://doi.org/10.1186/s12861-016-0129-6.

Walne P.R. (1966) Experiments in the large-scale culture of the larvae of *Ostrea edulis* L. In Fishery Investigations Series II; Ministry of Agriculture, Fisheries and Food: London, UK.

Ward J.E., Shumway S.E. (2004) Separating the grain from the chaff: particle selection in suspension- and deposit-feeding bivalves. J. Exp. Mar. Biol. Ecol. 300:83–130.

Webb S., Fidler A., Renault T. (2007) Primers for PCR-based detection of ostreid herpes virus-1 (OsHV-1): Application in a survey of New Zealand molluscs. Aquaculture. 272. 126-139. 10.1016/j.aquaculture.2007.07.224.

Xu H.S., Roberts N., Singleton F.L., Attwell R.W., Grimes D.J., Colwell R.R. (1982) Survival and viability of nonculturable*Escherichia coli* and*Vibrio cholerae* in the estuarine and marine environment 8: 313. https://doi.org/10.1007/BF02010671

Zhang X.J., Qin G.M., Bing X.W., Yan B.L. and Liang L.G. (2011) Molecular and phenotypic characterization of *Vibrio aestuarianus*, a pathogen of the cultured tongue sole, *Cynoglossus semilaevis* Gunther. Journal of Fish Diseases,34, 57-64.